Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendments, claims 1 - 36 are pending in the application, with claims 4-12, 34 and 35 being withdrawn from consideration due to a restriction requirement. Claims 1, 13, 14, 30, and 31 have been amended. Support for the amendment to claim 1 can be found in the specification at, *e.g.*, page 14, lines 10-13. Support for the amendment to claims 13 and 30 can be found in the specification at, *e.g.*, page 31, lines 5-21. Support for the amendment to claims 14 and 31 can be found in the specification at, *e.g.*, page 32, lines 1-8 and original claims 14 and 31. There is no issue of new matter.

It is believed that the amendments to the claims will put the case in condition for allowance or in better condition for appeal. 37 C.F.R. § 1.116(a). Thus, it is respectfully requested that these amendments be entered.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Summary of the Invention

The claims are directed to cell-specific and/or tumor-specific herpes viral mutants (or methods utilizing said mutants) comprising, at a minimum, a mutation in the gene encoding γ34. 5 and an insertion of at least one copy of the γ34. 5 gene under the transcriptional control of a cell specific and/or tumor specific promoter.

Claim Objections

The Examiner has maintained the objection of claims 13 and 30 "because they contain acronyms for genes/promoters which are not specifically recited in the specification and that they are well recognized in the art."[1] Paper No. 17, page 3. The Examiner states that the portions of the specification on page 31 that were noted by Applicants in the last Response do not specifically define the abbreviated terms but rather provide a first presentation of the acronyms. The Examiner also states that "while the claims are read in light of the specification, the metes and bounds of the claims should be defined within the recitation of the claim and be interpreted and stand by themselves. In this case, because the specification does not specifically define the abbreviations as encompassing any specific term, the first presentation of the abbreviated term should be denoted by setting forth the full name indicating the term to be used subsequently. *Id.* at pages 3-4.

While Applicants disagree with the rationale of this objection, solely in an effort to expedite allowance, Applicants have amended claims 13 and 30 to recite the full name of the acronyms AFP, CEA, and PSA. Withdrawal of this objection is respectfully requested.

Biological Deposit

The Examiner had previously rejected claims 15, 24, and 32 under 35 U.S.C. § 112, first paragraph, as "containing subject matter which was not described in the specification in

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[1]Applicants are not clear regarding the Examiner's statement "and that they are well recognized in the art." Should this objection be maintained, clarification is respectfully requested.

such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention" (Paper No. 14, pages 4-6). Specifically, the Examiner contended that since the Myb34.5 vector is not obtainable or available, the requirements of 35 U.S.C. § 112 regarding "how to make" may be satisfied by a deposit of the Myb34.5 vector. In their Reply filed January 27, 2003, Applicants provided assurance that they will make a deposit of the Myb34.5 vector that is acceptable for patent purposes and that complies with 37 C.F.R. §§ 1.801-808. In addition, after the deposit is made, Applicants stated that they will amend the specification to reflect the deposit, in accordance with 37 C.F.R. §§ 1.809 (d) and (e).

Applicants attach herein a copy of a receipt from the ATCC, dated June 16, 2003, indicating that herpes simplex virus type 1: Myb 34.5 was deposited pursuant to the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposit was received at the ATCC on January 28, 2003 and given patent deposit designation PTA-4963. A copy of the receipt is attached as **Exhibit A**. In accordance with 37 C.F.R. §§ 1.809 (d) and (e), the specification has been amended herein to reflect the deposit.

Applicants respectfully request that the Examiner acknowledge in writing that the biological deposit has now been made.

Rejections Under 35 U.S.C. § 112, first paragraph

At pages 5-10 of Paper No. 17, the Examiner rejects claims 1-3, 13, 16-23, 25-33, and 36 under 35 U.S.C. § 112, first paragraph, because the specification does not reasonably

provide enablement for the treatment of neoplastic cells (other than CNS, and metastatic liver and colon cancer cells), as well as promoters (other than the B-myb promoter). The Examiner concludes that the specification does not enable any person skilled in the art to practice the invention commensurate in scope with these claims. Applicants respectfully traverse this rejection.

Request for Clarification of Rejected Claims

With regard to claims 14 and 15, which recite that the promoter is "B-myb," Applicants had previously argued that, at the very least, these two claims should not be subject to this rejection. In response, the Examiner agreed that claim 15 should not be rejected (Paper No. 17, page 6, lines 13-14), but that

> claim 14 is broader [than] the specific B-myb promoter disclosed and encompasses any promoter derived from the gene that encodes B-myb. The specification provides only one specific B-myb promoter which is demonstrated to [be] functional and useful, and is silent with respect to any modified forms of this specific sequence, or to methods of or guidance for modifying this promoter sequence to "derive" a promoter for use in the methods or in the generation of useful products.

Id. at lines 14-19.

Thus, while the Examiner appears to have set forth, in Paper No. 17, arguments in support of a rejection of claim 14, Applicants note that claim 14 was *not* included in the list of rejected claims at the beginning of the rejection (*see*, Paper No. 17, page 5, line 1), but was included as a rejected claim on the Office Action Summary (PTO-326). Applicants have presumed that claim 14 was intended to be rejected, and have amended the claim for clarity (*i.e.*, deleted the term "derived"). If this is not the case, then Applicants respectfully request

that they be contacted immediately. It is believed that amended claim 14 is sufficiently enabled and that this rejection should be withdrawn.

Further in this regard, it is noted that claim 32 (directed to a method wherein the herpes mutant is Myb34.5) *was* included in the list of rejected claims at the beginning of the rejection (*see*, Paper No. 17, page 5, line 1). This is inconsistent with the withdrawal of the rejection of [similar in scope] claim 15. Further, claim 32 is not listed as a rejected claim on the Office Action Summary (PTO-326). Clarification is respectfully requested.

The Legal Standard for Enablement

In order to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, the claimed invention must be enabled so that any person skilled in the art can make and use the invention without undue experimentation. *See, In re Wands*, 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Applicants assert that it would require no more than routine experimentation for a skilled artisan to practice the full scope of the presently claimed invention in view of the teachings and guidance in the specification and the knowledge available in the art.

The PTO bears the initial burden of proving that a specification is non-enabling. *See, In re Marzocchi*, 169 USPQ 367 (C.C.P.A. 1971). It is axiomatic that a specification is presumed to be enabling unless the PTO provides acceptable objective evidence or sound scientific reasoning showing that it would require undue experimentation for one of ordinary skill in the art to make and use the claimed invention. Moreover, to enable a claimed invention, a specification need not teach, and preferably omits, information that is well-known to those of ordinary skill in the art. *See Hybritech Inc. v. Monoclonal Antibodies,*

Inc., 802 F.2d 1367, 1384 (Fed. Cir. 1986); *In re Wands*, 8 U.S.P.Q.2d 1400, 1402 (Fed. Cir. 1988). One of ordinary skill in the art is also deemed to know not only what is considered well-known, but also where to search for any needed starting materials. *See, In re Howarth*, 210 U.S.P.Q. 689, 692 (C.C.P.A. 1981). Finally, the test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, it is undue. *In re Angstadt*, 190 U.S.P.Q. 214, 219 (C.C.P.A. 1976).

The Present Specification Enables the Full Scope of the Claimed Invention

As Applicants understand it, the enablement rejection is directed to the scope of the "neoplastic cell" and/or the scope of the "cell-specific or tumor-specific promoter" recited in the claims. Pages 6-10 of Paper No. 17 detail the Examiner's position.

Regarding cell-specific promoters, the Examiner states:

> With respect to any other promoter, there is no guidance for the use of cell-specific promoter [sic] with respect to the ability of the HSV vectors killing a specific tissues [sic]. Further, while certain genes are known to be cell specific, their promoters out of context of the gene/genome do not necessarily provide tissue specific expression. However, while Examiner would agree that providing a cell specific promoter could potentially kill a specific tissue, the specification is silent with respect to why a normal tissue would be targeted by the instantly claimed vectors.

Paper No. 17, pages 6-7.

Applicants respectfully direct the Examiner's attention to page 29, line 19, to page 30, line 24, of the present specification, which states:

Exemplary cell-specific promoters include the following: endothelial nitric oxide synthase (eNOS) promoter expressed in endothelial cells (Guillot, P.V., *et al., J. Clin. Invest. 103*:799-805 (1999)); vascular endothelial growth factor (VEGF) receptor (flk1) promoter expressed in endothelial cells (Kappel *et al. Blood 93*: 4282-4292 (1999)); insulin promoter expressed in beta cells of the pancreas (Ray *et al., J. Surg. Res. 84*: 199-203 (1999)); promoter of gonadotropin-releasing hormone receptor gene expressed in cells of the hypothalamus (Albarracin *et al., Endocrinology 140*: 2415-2421 (1999)); matrix metalloproteinase 9 promoter, expressed in osteoclasts and keratinocytes (Munant *et al., J. Biol. Chem. 274*: 5588-5596 (1999)); promoter of parathyroid hormone receptor expressed in bone cells (Amizuma *et al., J. Clin. Invest. 103*: 373-381 (1999)); and dopamine beta-hydroxylase promoter expressed in noradrenergic neurons (Yang *et al., J. Neurochem. 71*: 1813-1826 (1998)).

Cell-cycle regulated promoters are also a type of cell-specific promoter. Other cell-specific promoters will be known to those skilled in the art.

Applications of this vector embodiment include the elimination of select noxious cell populations in an organ, as well as animal models to study the elimination of select populations in an organ during development. Exemplary applications of this embodiment include the following:

1) Treatment options to eliminate a noxious cell population: In one example, in conditions where there is exuberant neovascularization of blood vessels, such as cerebral Moya-Moya disease, the use of the flk1receptor promoter to drive gamma 34.5 gene expression would allow for selective elimination of the blood vessels causing this disease.

In another example, in conditions where there is extensive bone remodeling and elimination of bone, such as osteoporosis, the use of the matrix metalloproteinase 9 or the parathyroid hormone receptor to drive expression of gamma 34.5 would eliminate bone osteoclasts from further remodeling of bone.

2) To study the effect of elimination of select populations in an organ during development: For example, in order to study the effect of elimination of a cell population on developmental processes, one could use, for example, the dopamine-beta-hydroxylase promoter to eliminate the

> noradrenergic neurons and then study the effect on animal development.

Thus, contrary to the Examiner's assertion, the present specification does, in fact, provide guidance and examples on how those skilled in the art would use cell-specific promoters in the claimed HSV mutants to target and eliminate a particular cell population. The Examiner has not provided acceptable objective evidence or sound scientific reasoning to show that it would require undue experimentation for one skilled in the art to practice this aspect of the invention.

Regarding cancer related genes and use of their promoters, the Examiner states:

> each of these promoters provides expression in cells other than just tumors. For example, PSA is normally expressed by the prostate as well as erb-B2, tyrosinase and MUC1. While CEA and AFP are cancer associated antigens in adults, the promoters when provided in the context of a transgene will be expressed in a wide variety of cells, transformed and nontransformed.

Paper No. 17, page 7.

While it is possible for some level of expression of tumor associated antigens in normal cells, the claims do not require 100% cell- or tumor- specific targeting. The herpes mutants of the invention *preferentially* target and *preferentially* replicate in specific cell types or tumor types as a result of the herpes γ34.5 gene (a gene required for efficient *in vivo* viral replication) being deleted and reintroduced under the transcriptional control of a cell- or tumor- specific promoter. The cell- or tumor- specific promoter functions *preferentially* in the corresponding cell or tumor, so that there is selective or preferential targeting and viral replication, with minimum effect on non-target or non-tumor tissue.

With respect to the scope of enablement of the claimed invention, the Examiner maintains that herpes vectors are known only to infect cells of the central nervous system and metastatic cells of liver and colon origin. *See*, Paper No. 17, page 7. As set forth in the last Reply, Applicants respectfully disagree with this statement. Herpes viruses, and especially, HSV-1 and HSV-2, are widely known to infect virtually *all* vertebrate cell types. (*See*, specification, page 26, lines 20-27). The Mullen *et al.* paper (already of record) shows that HSV can infect breast and pancreatic cancer cells. Wu *et al.*, *Cancer Res. 61*:3009-3015 (April 2001)(copy attached as **Exhibit B**), shows that herpes simplex virus can be used to purge human bone marrow cells of breast cancer cells. Clearly, HSV can infect cells other than CNS and metastatic liver and colon cells. Moreover, the tumor-specific promoter or cell-specific promoter used in the herpes viral construct of the invention to drive expression of the γ34.5 gene will give the herpes virus the selective capability of targeting and killing the type of cells that overexpress the tumor-specific or cell-specific protein corresponding to the promoter present in the herpes construct.

The Examiner states that "the mere recitation of promoters which broadly meet the functional language in light of their endogenous expression does not provide for their use in the artificial context of an HSV vector" and that the specification is silent regarding other promoters, besides the B-myb promoter, to affect expression in neoplastic cells. *Id.* Further, the Examiner states that the specification does not provide specific guidance on what portions of any other particular promoter one should use and in what types of neoplastic cells one should use said promoters.

Contrary to the Examiner's assertion, Applicants have provided considerable direction and guidance on other promoters (besides B-myb) that may be used in the vectors and

methods of the invention as well as in what types of neoplastic cells one should use them. The Examiner's attention is directed to the present specification, at, *e.g.*, page 16, line 6, to page 17, line 2, page 17, line 27, to page 18, line 14, page 30, line 25, to page 32, line 16. Below is a representation of the guidance provided in this regard on the above-referenced pages of the specification:

DF3 (MUC1) (which is overexpressed in the majority of breast cancers)

AFP (which is overexpressed in hematoma)

CEA (which is overexpressed in gastrointestinal and lung cancers)

PSA (which is overexpressed in prostate cancers)

tyrosinase (which is overexpressed in melanomas)

c-*erb*B2 (oncogene overexpressed in breast, pancreatic, ovarian, or gastric carcinomas)

Tumor cells are also known to overexpress particular oncogenes, so that cells with upregulated gene expression can be targeted using the promoter elements of such genes. B-*myb*, C-*myb*, c-*myc*, c-*kit*.

Tumor- specific promoters were well-known and well-characterized in the art at the time of the present invention. Their DNA sequences were available in scientific papers or the GenBank sequence database. To aid the skilled artisan, Applicants provided reference citations for every promoter recited, as well as for viral tumor targeting generally. *See, e.g.*, specification, page 29, lines 1-10; page 31, line 5, to page 32, line 16. Furthermore, the present specification teaches the construction of an exemplary herpes viral mutant, Myb34.5, and how to use it to kill both neoplastic cells of the CNS (Example 1) as well as the periphery (Example 2).

In Applicants' working examples, the herpes viral mutant utilized contained the promoter of an oncogene, "B-myb," that is overexpressed in many different types of tumors[2], and accordingly, can be used in many different tumor types. *See*, Examples 1 and 2 of the present specification, which clearly demonstrate applicability of a herpes mutant containing B-myb in both CNS tumors and peripheral tumors (colon carcinoma and metastatic liver cancer).

Applicants respectfully remind the Examiner that independent method claim 17 recites in part: "A method for selectively *killing neoplastic cells that overexpress a known tumor-specific protein*", and that part (b) of claim 17 recites that the γ34.5 gene is *"under the transcriptional control of the promoter of said tumor-specific protein"*, such that said promoter drives expression of said γ34.5 gene. (Emphasis added). Thus, the claim is written so that there is a clear rationale and nexus, in the claims, between the promoter chosen and the type of neoplastic cell targeted.

Thus, rather than limit the claims to the particularly exemplified promoter (*i.e.*, B-myb), and the particularly exemplified neoplastic cells (CNS tumors, colon carcinoma and metastatic liver cancer), Applicants respectfully request that the Examiner reconsider his position based on the fact that one skilled in the art would appreciate which promoter to select based on the type of neoplastic cell or cell-type being targeted. Considerable direction and guidance is clearly set forth in the present specification (*i.e.*, pages 29-32), and coupled

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[2]As discussed in the specification at page 69, lines 16-28, the DNA sequence of the B-myb promoter, as well as the DNA sequence of several other promoters, contains regions that bind the transcription factor E2F. E2F is regulated by the cell-cycle-regulatory p16/retinoblastoma/cdk4 pathway. In normal, quiescent cells, where this pathway is active, E2F is shut off and the B-myb promoter is shut off as well. In cycling cells or tumor cells, where this pathway is inactive, E2F is active and the B-myb promoter is active as well. The p16/retinoblastoma/cdk4 pathway has been estimated to be defective or altered in greater than 90% of cancers.

with the knowledge and information available to the skilled artisan, would possibly require the practice of routine, but not undue, experimentation. Moreover, as discussed above, and absent objective evidence to the contrary by the Examiner, the use of the B-myb promoter is useful broadly in most all types of tumor cells. The same rationale applies to claim 33, which is directed to "A method for selectively eliminating a target cell population that overexpresses a known cell-specific protein."

The above notwithstanding, in addition to the specification, Applicants have previously provided post filing date art (Mullen) which utilized other tumor specific promoters than those exemplified in the specification. Even more promoters were used, vectors engineered, and experimental results provided in the attached Declaration Under 37 C.F.R. § 1.132 of Dr. E. Antonio Chiocca (a co-inventor). This will be discussed in greater detail below.

In response to Applicants' assertions in the last Reply that those skilled in the art would appreciate, that the claims directed to the herpes viral mutant can also be used for *in vitro* determination of a given mutant virus's oncolytic efficacy or cytotoxicity, in effect, as a screening tool prior to *in vivo* use (specification, Examples 1 and 2), the Examiner contends that this "is a circular argument which affirms the only use of the vectors is for *in vivo* therapy." Paper No. 17, page 8. Applicants respectfully disagree.

One skilled in the art would appreciate that such a *in vitro* "screening tool" would be very valuable in order to determine, prior to therapy, if *in vivo* treatment would even be beneficial. For example, a tumor sample (biopsy) could be subject to one or more of the HSV mutants of the invention *in vitro*, and viral replication in the tumor cells assayed.

Further, Applicants respectfully remind the Examiner that in addition to the above mentioned *in vitro* use, Applicants also stated, in the last Response, that one skilled in the art would also appreciate the claimed herpes viral mutant's use, *in vitro*, in functional studies of, *i.e.*, the γ34.5 gene. This was not addressed by the Examiner. Applicants maintain that one skilled in the art would certainly appreciate these and other *in vitro* applications of the claimed vectors.

Further in this regard, the Examiner states that "the specification does not teach that the vectors should be used or would be useful in killing cells *in vitro* for any purpose. Killing cells *in vitro* with HSV vectors is not a readily apparent use recognized in the art, and contrary to Applicants' arguments the specification is silent to any apparent use *in vitro* for the instantly claimed products." Paper No. 17, page 8. Applicants submit that a representative *in vitro* use was addressed above and that others would be apparent to those skilled in the art.

Absent acceptable objective evidence or sound scientific reasoning to doubt these assertions, the present specification must be considered enabling under *Marzocchi*.

The Mullen et al. Paper Further Supports a Broad Scope of Enablement for the Claimed Invention

In the last Reply, to support enablement of the full scope of the claimed invention, especially in terms of the type of neoplastic cell as well as the type of tumor-specific or cell-specific promoter, Applicants submitted and discussed the post-filing date reference of Mullen *et al.*, "Regulation of Herpes Simplex Virus 1 Replication Using Tumor-Associated Promoters," *Annals of Surgery 236*:502-513 (2002). Mullen used Applicants' teachings as

guidance[3] to construct HSV mutants wherein two different tumor-specific promoters (CEA and DF3) were used to drive γ34.5 expression. Selective *in vitro* killing of tumor cells (breast, colon, and pancreatic), which were known to overexpress the corresponding tumor-associated proteins, was shown.

Mullen demonstrated that regulation of γ 34.5 expression by either the CEA promoter or the MUC1/DF3 promoter during HSV-1 infection modulates viral replication, with preferential replication in cells that overexpress the corresponding tumor-associated antigen (*See*, Mullen, pages 507-508). Moreover, in an *in vivo* study, a single intratumoral inoculation of an HSV-1 mutant with the MUC1/DF3 promoter regulating γ34.5 expression results in significant antineoplastic activity in MUC1-positive pancreatic carcinoma xenografts as compared to mock inoculation. (*See*, Mullen, page 508, right column, and Figure 4B).

Very significantly, Mullen states:

> We chose the same DF3 promoter sequence [as Kurihara *et al., J. Clin. Invest. 106*:763-771 (2000)] to regulate $\gamma_1$34.5 expression in the HSV-1 mutant DF3γ34.5. In addition, $\gamma_1$34.5 gene expression and HSV-1 replication have been regulated by a cell cycle-dependent B-myb promoter in the HSV-1 mutant Myb34.5 [citing to the paper by Chung *et al.*, which corresponds to the present invention]. We have *extended these results* in our construction of DF3γ34.5 by demonstrating preferential HSV-1 replication of DF3γ34.5 in MUC1-positive cells and inhibition of tumor growth. The magnitude of tumor growth inhibition is similar to that observed with. . . the oncolytic HSV-1 mutant Myb34.5. Emphases added.

Mullen, page 511, left col., lines 11-22.

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[3]Applicants note that reference 18 in the Mullen paper is Chung *et al., J. Virol. 73*:7556-7564 (1999), which corresponds to the subject matter of the present invention.

However, on page 8 of Paper No. 17, the Examiner states that while the vectors taught in Mullen are encompassed by the present claims, Mullen *et al.* shows that "the MUC-1 promoter provides expression in a range of cells and provided expression and killed both normal and transformed cells (page 508, middle of second column)." Applicants respectfully submit that the Examiner has misinterpreted the Mullen results and reconsideration based on the following remarks is respectfully requested..

In replication assays, Mullen shows that in MUC-1 negative cell lines (*i.e.*, A375, HUVEC, and Vero[4]), viral replication of DF3γ34.5 was as attenuated as the MGH1 virus[5]. *See*, Mullen page 508, right column, first full paragraph, and Figure 3C, last 3 sets of columns. In contrast, in MUC-1- positive cancer cell lines (*i.e.*, MCF7, CAPAN2, and SW1990[6]), viral replication of DF3γ34.5 was as robust as that of hrR3 in SW1990 cells and 1-2 log orders greater than MGH1 in all of the MUC1-positive cell lines. *See*, Mullen page 508, right column, first full paragraph, and Figure 3C, first 3 sets of columns.

Further, in cytotoxicity assays *in vitro*, the cytotoxicity of DF3γ34.5 against the MUC-1 negative cell lines A375 and HUVEC was attenuated compared to hrR3. The cytotoxicity of DF3γ34.5 against the MUC-1 negative Vero cells was similar to that of MGH1. *See*, Mullen page 508, right column, second full paragraph, and Figure 4A (page 509, left half of Figure). The cytotoxicity of DF3γ34.5 against all three MUC-1 positive cell

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[4]A375 are human melanoma cells; HUVEC are normal endothelial cells; and Vero cells are African green monkey kidney cells.

[5]MGH1 is defective in both γ34.5 and ICP6 expression.

[6]SW1990 are human pancreatic carcinoma cells; MCF7 are human breast carcinoma cells; and CAPAN2 are human pancreatic carcinoma cells.

lines (*i.e.*, MCF7, CAPAN2, and SW1990) was similar to that of hrR3. *See*, Mullen page 508, right column, second full paragraph, and Figure 4A (page 509, right half of Figure).

Finally, in *in vivo* studies of DF3γ34.5, CAPAN2 tumors (MUC-1 positive human pancreatic cancer cells) were implanted into the flanks of nude mice. The tumors were treated with a single direct intratumoral inoculation of DF3γ34.5 or heat-inactivated DF3γ34.5. The tumors injected with heat-inactivated virus grew rapidly, whereas tumor growth was significantly inhibited following DF3γ34.5 injection. *See*, Mullen page 508, right column, third full paragraph, and Figure 4B (page 510).

On pages 8-9 of Paper No. 17, the Examiner states that the methods of *in vivo* delivery in Mullen *et al.* are direct injection into a tumor, wherein Applicants assert that the HSV vectors can be delivered generally, and will be selectively found/expressed by virtue of the tissue/tumor-specific promoter. The Examiner concludes that the ability of the vector to be expressed in normal and transformed cells [as shown allegedly by Mullen] teach that tumor promoters require specific delivery methods. Applicants respectfully disagree.

As discussed above, while it is possible for some level of expression of tumor associated antigens in normal cells, the claims do not require 100% cell- or tumor- specific targeting. The herpes mutants of the invention *preferentially* target and *preferentially* replicate in specific cell types or tumor types as a result of the herpes γ34. 5 gene (required for viral replication *in vivo*) being deleted and reintroduced under the transcriptional control of a cell- or tumor- specific promoter. The cell- or tumor- specific promoter functions *preferentially* in the corresponding cell or tumor, so that there is selective or preferential targeting and viral replication, with minimum effect on non-target or non-tumor tissue. This was fully supported by the teachings in Mullen, as clarified above. The Examiner has not

provided objective evidence to the contrary that systemic delivery would not demonstrate selective targeting.

Clearly, the Mullen paper supports the scope of the present claims beyond the particularly exemplified B-myb promoter and neoplastic cell-types. Applicants respectfully request reconsideration of the Mullen paper based on the above remarks.

The Declaration Under 37 C.F.R. § 1.132 Further Supports Enablement of the Claimed Invention

Finally, Applicants submit herewith a Declaration Under 37 C.F.R. § 1.132, executed by one of the co-inventors, Dr. E. Antonio Chiocca, presenting the results of *in vitro* and *in vivo* studies using two additional combinations of promoters and tumor cells. As explained in greater detail in the Declaration, two HSV mutants were engineered: one, having the γ34.5 gene under the transcriptional control of nestin, a glioma-specific promoter, and the other, having the γ34.5 gene under the transcriptional control of the PSA promoter, a prostate cancer-specific promoter. The HSV mutant with one copy of the γ34.5 gene driven by the nestin promoter replicated and lysed glioma cells more efficiently than controls and with a larger therapeutic index than wild-type HSV1 (*see*, Table 1 in the Declaration). The HSV mutant with one copy of the γ34.5 gene driven by a PSA promoter replicated and lysed prostate cancer cells more efficiently than controls.

In summary, Applicants respectfully submit that a skilled artisan, in view of the direction and guidance provided in the specification, the knowledge generally available and known in the art, the confirmatory teachings of Mullen *et al.*, as well as the experimental data provided in the attached Declaration of Dr. Chiocca, which employed different promoter/tumor cell combinations than Applicants, would have been able to make, use and

practice the full scope of the claimed herpes viral mutant, methods of selectively killing neoplastic cells, and methods for selectively eliminating a target cell population, without undue experimentation. The Examiner has failed to provide any sound evidence or scientific reasoning as to why the specification would not enable the full scope of the claimed invention. Thus, a *prima facie* case of lack of enablement has not been established. Applicants therefore respectfully request that this rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all currently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned directly at (202) 772-8637.

Prompt and favorable consideration of this Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Karen R. Markowicz

Karen R. Markowicz
Agent for Applicants
Registration No. 36,351

Date: 11/19/03

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Exhibit-A

Exhibit A

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-365-2745

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Massachusetts General Hospital
Attn: E. Antonio Chiocca
Molecular Neuro-Oncology Lab
13th Street, Building 149 Rm 6119
Charlestown, MA 02129

RECEIVED
JUN 18 2003
STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

TWJ KRM 6/18 6/18/03 KRM

Deposited on Behalf of: Massachusetts General Hospital

Identification Reference by Depositor:	**Patent Deposit Designation**
Herpes Simplex Virus Type I: Myb 34.5	PTA-4963

The deposit was accompanied by: __ **a scientific description** _ **a proposed taxonomic description indicated above.**

The deposit was received January 28, 2003 **by this International Depository Authority and has been accepted.**

AT YOUR REQUEST: X **We will inform you of requests for the strain for 30 years.**

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested May 14, 2003. **On that date, the culture was viable.**

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Marie Harris

Marie Harris, Patent Specialist, ATCC Patent Depository

Date: June 16, 2003

cc: Karen Markowicz
Ref: Docket or Case No.: 0609.4880002/JAG/KRM

Exhibit-B

Exhibit B

[CANCER RESEARCH 61, 3009–3015, April 1, 2001]

Biological Purging of Breast Cancer Cells Using an Attenuated Replication-competent Herpes Simplex Virus in Human Hematopoietic Stem Cell Transplantation[1]

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ABSTRACT

Autologous hematopoietic stem cell transplantation after myclosuppressive chemotherapy is used for the treatment of high-risk breast cancer and other solid tumors. However, contamination of the autologous graft with tumor cells may adversely affect outcomes. Human hematopoietic bone marrow cells are resistant to herpes simplex virus type 1 (HSV-1) replication, whereas human breast cancer cells are sensitive to HSV-1 cytotoxicity. Therefore, we examined the utility of G207, a safe replication-competent multimutated HSV-1 vector, as a biological purging agent for breast cancer in the setting of stem cell transplantation. G207 infection of human bone marrow cells had no effect on the proportion or clonogenic capacity of $CD34^+$ cells but did enhance the proliferation of bone marrow cells in culture and the proportion of $CD14^+$ and $CD38^+$ cells. On the other hand, G207 at a multiplicity of infection of 0.1 was able to purge bone marrow of contaminating human breast cancer cells. Because G207 also stimulates the proliferation of human hematopoietic cells, it overcomes a limitation of other purging methods that result in delayed reconstitution of hematopoiesis. The efficient infection of human bone marrow cells in the absence of detected toxicity suggests that HSV vectors may also prove useful for gene therapy to hematopoietic progenitor cells.

INTRODUCTION

Approximately 6–9% of white women in Europe and North America will develop breast cancer in their lifetime (1). Despite progress made over the last decades in the diagnosis and treatment of breast cancer, overall survival has not significantly improved, with the median 10-year survival <50%. Intensive, multiagent chemotherapy, "adjuvant therapy," has become a standard for treatment (2). Unfortunately, bone-marrow toxicity is the major confounding factor and limitation in dose escalation. Therefore, high-dose chemotherapy has been followed by autologous bone marrow or PBPC[3] transplantation (3–6). Contamination of bone marrow or peripheral blood with breast cancer cells is common, ranging from approximately 30 to 80% in bone marrow and 10 to 55% in PBPC, depending on the stage of disease and detection technique (7–11), and may be a highly significant predictor of relapse (12, 13). There is some uncertainty about the correlation between tumor cell contamination of autologous stem cell products and survival. However, patients with significant tumor cell contamination are often excluded from autologous bone marrow transplant protocols. Inferior outcomes occur when tumor cell-contaminated autologous bone marrow is infused after myeloblative chemotherapy (11, 14, 15). In contrast, no association between occult tumor contamination and overall survival has been found in patients receiving PBPCs (16, 17).

Ex vivo purging of tumor cell-contaminated bone marrow or PBPCs before transplantation may enhance the efficacy of autologous hematopoietic stem cell transplant if toxicity to stem cells can be minimized. A number of approaches have been described for purging bone marrow of breast cancer cells, including *ex vivo* chemotherapy (9, 18), $CD34^+$ cell-enrichment (9, 19), immunotoxins (20–23), immunomagnetic removal (22, 24), and adenovirus vectors expressing wild-type p53 (25) or "suicide" genes (26, 27).

Attenuated, replication-competent HSV vectors are an attractive strategy for tumor therapy because mutant viruses are available that replicate in dividing cells with consequent cell death and *in situ* viral spread but are incapable of replication in normal tissue (28, 29). We have constructed such a multimutated HSV-1 vector, termed G207, containing deletions of both $\gamma 34.5$ loci, the major viral determinant of neurovirulence (30), and an *E. coli lacZ* insertion that inactivates the *ICP6* gene, encoding the large subunit of ribonucleotide reductase (31, 32). G207 is efficacious in the treatment of multiple human tumors in athymic mice (32–34), including breast cancer (35), and mouse tumors in syngeneic animals (36, 37). However, G207 is nonpathogenic in HSV-sensitive mice and nonhuman primates (38, 39), and no toxicity has been observed after intracerebral inoculation in a Phase I clinical trial for the treatment of recurrent malignant glioma (40). Human bone marrow cells, both proliferating and nonproliferating cells, are very resistant to HSV cytotoxicity. The different susceptibilities of bone marrow and breast cancer cells to G207 prompted us to investigate the use of G207 to purge contaminating breast cancer cells from human bone marrow in the setting of autologous stem cell transplantation.

MATERIALS AND METHODS

Cells and Viruses. Human bone marrow was obtained by flushing the collection bags used for harvesting marrow from normal donors. The Department of Health and Human Services/NIH guidelines on protection of human subjects were followed, with the authorization of the Georgetown University Institutional Review Board. Bone marrow was diluted in PBS (Life Technologies, Inc.), layered on Fico/Lite-LymphoH (Atlanta Biologicals, Atlanta, GA), and centrifuged for 30 min at 900 × *g*. The buffy coat was collected and washed twice with RPMI 1640 (Life Technologies, Inc.) containing 10% FCS (BioFluids Inc., Rockville, MD). The MNC were adjusted to 10^6 cells/ml in MyeloCult H5100 long-term culture medium (Stem Cell Technologies Inc., Vancouver, British Columbia, Canada).

Human breast cancer cell lines MDA-MB-231 (41) and MDA-MB-435 (42) and human chronic myelogenous leukemia cell line K562 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media supplemented with 10% FCS, glutamine, antibiotics, non-

Received 8/4/00; accepted 1/26/01.

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[1] Supported in part by NIH Grant RO1-NS32677 (to R. L. M.) and Department of Defense Grant DAMD17-99-1-9202 (to S. D. R.). Samuel D. Rabkin and Robert L. Martuza are consultants to NeuroVir, Inc., which has a license from Georgetown University for G207.

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[3] The abbreviations used are: PBPC, peripheral blood progenitor cell; HSV, herpes simplex virus; MNC, mononuclear cells; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; MOI, multiplicity of infection; CFU, colony-forming unit; p.i., post-infection; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; CFU-Tr; CFU-tumor cells; RT-PCR, reverse transcription-PCR; FACS, fluorescence-activated cell sorter; ICP, infected cell polypeptide.

essential amino acids, and sodium pyruvate. Vero (African green monkey kidney) cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT).

G207 is an HSV-1 mutant containing deletions in both copies of the *γ34.5* gene and an *E. coli lacZ* insertion inactivating the *ICP6* gene (32). Wild-type HSV-1 strain KOS was obtained from David Knipe (Harvard Medical School, Boston, MA). Virus stocks were prepared from low-multiplicity infections of Vero cells (39) and titered by plaque assay on Vero cells. Defective HSV vector dvHCL was generated using amplicon plasmid pHCL and helper virus HSV-1 tsK as described (43).

Viral Infection of Human Bone Marrow and Tumor Cells. Bone marrow or K562 cells were pelleted in 15-ml Falcon tubes (Becton Dickinson Labware, Franklin Lakes, NJ) and then mixed with G207 or KOS in 0.2 ml of PBS/1% heat-inactivated FCS at the MOI indicated or with vehicle alone (mock). The tubes were intermittently rocked for 45 min and incubated for an additional 60 min at 37°C, and the viral supernatants were removed by centrifugation. Infected bone marrow cells were resuspended in MyeloCult medium supplemented with 20 ng/ml GM-CSF, 10 ng/ml IL-3, and 10 ng/ml stem cell factor (R&D Systems, Inc., Minneapolis, MN) at 10^6 cells/ml or RPMI 1640 media supplemented with 10% FCS and cultured at 37°C. Infected K562 cells were resuspended in RPMI 1640 with 10% FCS. MDA-MB-231 and MDA-MB-435 were grown to subconfluence in 25 cm^2 tissue culture flasks and then infected with HSV-1. Viable cell numbers were determined by counting trypan blue (Sigma Chemical Co., St. Louis, MO) excluding cells on a hemocytometer.

G207 or dvHCL-infected cells were detected by X-gal histochemistry. Bone marrow cells were pelleted, fixed with cold 2% formaldehyde/0.2% glutaraldehyde, and stained with X-gal solution containing 1 mg/ml X-gal (Sigma Chemical Co.), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS for 4–24 h at 37°C. Images were scanned from slides (Polaroid SprintScan 34), and figures were prepared in Adobe Photoshop.

Flow Cytometry. Two-color immunofluorescent staining was used to analyze the bone marrow cells (FACS analysis). MNCs obtained from bone marrow were resuspended in PBS containing 0.3% BSA, 0.25% human globulin, and 0.3% sodium azide at a concentration of 10^6 cells/0.1 ml and incubated for 15 min at 25°C, and aliquots of 5×10^5 cells were stained with monoclonal antibodies directed against human CD34, CD38, CD3, CD14, CD19, and CD45 (Becton Dickinson, San Jose, CA). Cell analysis was performed on FACStar^{plus} (Becton Dickinson). CD45, a pan leukocyte marker, was used for selection of the gate. The data were analyzed with Reproman and Lysys II software. Cytokinetic analysis was performed with Vindelov's protocol (44). Briefly, cells were frozen in citrate buffer, thawed, treated with RNase A (Sigma Chemical Co.), stained with propidium iodide (Sigma Chemical Co.), and analyzed by FACS and Modfit software (Verity Software, Topsham, ME).

Clonogenic Assay. MNCs obtained from bone marrow were added to semisolid media containing 0.9% methylcellulose (methylcellulose ready mix without growth factors; HCC 4230; Stem Cell Technologies Inc.) supplemented with 2 units/ml erythropoietin and 20 ng/ml GM-CSF. One-ml cultures (10^5 cells) were set up in 35-mm Petri dishes in duplicate and incubated at 37°C in humidified 5% CO_2. Colonies (CFU-culture) were scored on day 14. Only colonies containing more than 40 cells were counted. Hemoglobinized colonies (red color) were considered as burst-forming unit-erythroid and the remainder as CFU-granulocyte/monocyte.

Bone Marrow Contamination with Breast Cancer Cells. Exponentially growing breast cancer cells were added to bone marrow cells at ratios of 1, 5, and 10%. The cell mixtures, in triplicate, were infected with G207 (MOI, 0.1) or vehicle alone (mock), incubated at 37°C, and harvested on days 3 and 6 after infection for determination of minimal residual disease. To detect minimal residual disease, contaminated bone marrow cells were added to semisolid media containing 0.9% methylcellulose without hematopoietic growth factors (10^5 cells/ml), and 1 ml was plated in 35-mm Petri dishes (two cultures/group). Under such conditions, all of the colonies would be derived from tumor cells. As confirmation, fresh bone marrow cells were added to the same semisolid media and plated. The plates were incubated at 37°C in humidified 5% CO_2 for 7 days, and the number of colonies was scored CFU-Tr.

Keratin-19 RT-PCR was a second assay for residual breast cancer cells. As a positive control, a skin biopsy was obtained from a breast cancer patient (with informed consent) and digested with 25 units/ml dispase (Life Technologies, Inc.) for 2 h at 37°C. Total RNA was extracted from normal bone marrow, bone marrow cells contaminated with breast cancer cells, breast cancer cells, and skin cells with TRIzol (Life Technologies, Inc.) according to the manufacturer's instruction. Total RNA (1 μg) in 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates, 10 mM DTT, 2.5 μg of oligo$(dT)_{12-18}$, and 10 units of RNase inhibitor was incubated with 200 units of reverse transcriptase (Superscript II; Life Technologies, Inc.) at 37°C for 60 min followed by 94°C for 5 min. Two μl of the cDNA was amplified in 50 μl of 20 mM Tris (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM deoxynucleotide triphosphates, 0.5 μM primers, and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). The keratin-19 primer sequences were 5′-ATCTTCCTGTCCCTCGAGCA-3′ (sense) and 5′-AGGTGGATTCCGCTCCGGGCA-3′ (antisense). The reaction was started at 94°C for 4 min, subjected to 35 cycles of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C, and finished at 72°C for 7 min. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.

RESULTS

G207 Enhances the Proliferation of Human Bone Marrow Cells. Infection of human bone marrow cells by G207 or wild-type HSV-1 KOS did not lead to any detectable decrease in the cell number in the first week after infection, even at an MOI of 3 (Fig. 1). Interestingly, by 2 weeks p.i. the number of viable cells in the G207-infected cultures was significantly greater than in the mock or KOS-infected cultures (Fig. 1; $P < 0.001$; Student's t test). The proliferative effect was somewhat dose dependent, because there was a larger increase in cell number in the cultures infected with a 10-fold higher dose of G207 (Fig. 1; MOI, 3). A similar 2–3-fold increase in cell number was seen when human bone marrow cells were cultured in conditioned media obtained from G207-infected bone marrow cultures at day 14 p.i. (data not shown).

The proportion of mitotically active cells in S + G_2-M phases was determined by flow cytometry (Table 1). The average proportion of mitotically active cells in six fresh human bone marrows was 15.3%, which decreased to 9.9% after 14 days in culture. In the HSV-infected cultures, the proportion did not decrease, whereas the G207-infected cells actually increased to 19.8% (Table 1; $P < 0.001$ compared with mock; Student's t test). The increase in S + G_2-M phase cells further illustrates the proliferative effect of G207 infection.

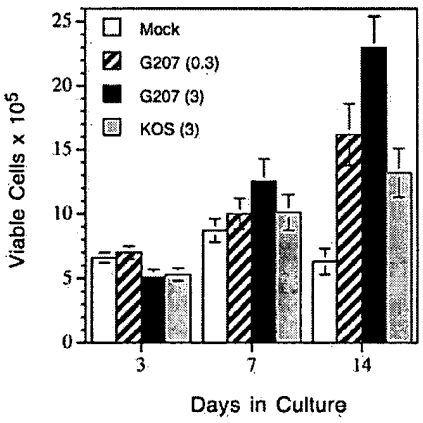

Fig. 1. Proliferation of human bone marrow cells after infection with HSV. Human bone marrow cells were infected with G207 or KOS at the MOIs indicated (in parentheses) on day 0 and cultured in long-term culture media supplemented with GM-CSF (20 ng/ml), IL-3 (10 ng/ml), and stem cell factor (10 ng/ml). Viable cell counts were determined by trypan blue exclusion on the days indicated. Values are the mean ± SE of triplicate samples. The number of cells at day 14 in the G207- (MOI, 3) infected cultures are significantly greater than the KOS-infected cells ($P < 0.05$; Student's t test) or G207 (MOI, 0.3) infected cells ($P < 0.01$; Student's t test).

HSV Infection of Human Bone Marrow Cells. To determine whether HSV was able to actually infect bone marrow cells, cells were infected with a defective HSV vector expressing lacZ driven by the CMV immediate-early promoter (dvHCL). Numerous X-gal positive cells were seen, some with very intense staining (Fig. 2; *dvHCL**) and others with low levels of staining (Fig. 2, *dvHCL*<). After G207 infection, only a small proportion of cells were stained with X-gal (Fig. 2; *G207*). G207 contains the *lacZ* gene driven by the *ICP6* promoter, a "leaky" early promoter (45), transactivated by ICP0 (31).

Table 1 *Cytokinetic analysis of human bone marrow cells*

Bone marrow was mock-, G207-, or KOS-infected (MOI = 0.1), and the stage in the cell cycle was determined by flow cytometry on the days p.i. indicated. Values are the mean percentage of cells in S + G_2/M phases ± SE (n = 3). On day 14, the mean percentage of S + G_2/M phase cells after G207 infection is significantly greater than for KOS ($P < 0.02$; Student's t test).

	Days p.i.			
Virus	0	3	7	14
Mock	15.2 ± 1.2	16.4 ± 2.4	15.4 ± 2.8	9.9 ± 0.9
G207		16.6 ± 3.2	18.9 ± 1.6	19.8 ± 1.2
KOS		18.9 ± 3.1	17.1 ± 2.2	16.1 ± 2.3

Table 2 *Phenotype of human bone marrow cells after 14 days in culture*

Cells were infected with G207 or KOS at a MOI of 0.1. At day 14, they were analyzed by FACS after staining for the following cell surface markers: CD34, a marker for hematopoietic precursor cells; CD19, pan B cell marker; CD3, T-lymphocyte receptor; CD14, a monocyte marker; and CD38, a nonlineage-restricted marker expressed on progenitor cells, plasma cells, B lymphocytes, and activated T lymphocytes. Values are the mean percentage of positive cells after gating ± SE ($n \geq 6$).

	Mock	G207	KOS
CD34	0.9 ± 0.2	0.8 ± 0.1	0.8 ± 0.1
CD19	0.7 ± 0.1	0.9 ± 0.2	0.8 ± 0.1
CD3	1.3 ± 0.1	2.0 ± 0.6	2.1 ± 0.2
CD14	10.3 ± 1.1	55.7 ± 5.2	62.6 ± 4.5
CD38	30.1 ± 2.1	73.6 ± 5.9	71.0 ± 6.2

We were unable to detect infectious virus in G207-infected bone marrow (MOI, 1 or 0.1) at day 6 p.i. (limit of detection was 10^2 plaque-forming units/ml). This suggests that the block to G207 replication occurs at an early step after infection.

HSV Infection Does Not Affect Hematopoietic Progenitors. The percentage of $CD34^+$ cells in fresh normal bone marrow was 2.1% as determined by flow cytometry. There was no expansion of $CD34^+$ cells in the HSV-infected bone marrow and no difference between G207- and mock-infected cultures in the proportion of $CD34^+$ cells

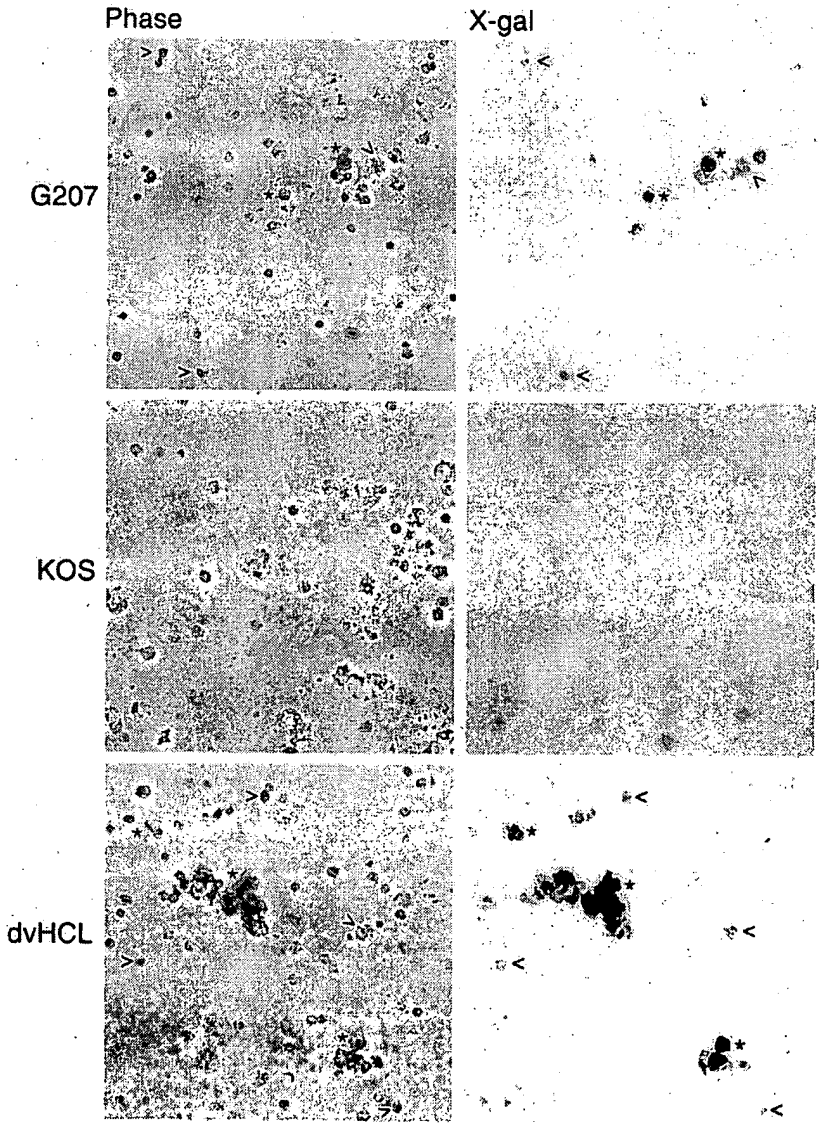

Fig. 2. Infection of human bone marrow cells by HSV. Human bone marrow cells were infected with G207 (*top*; MOI, 5), KOS (*middle*; MOI, 0.1), or dvHCL (*bottom*; MOI, 0.4), cultured in RPMI 1640 supplemented with 10% FCS, fixed on day 2 (G207, dvHCL) or day 3 (KOS) p.i., and processed for X-gal histochemistry. Images were photographed under phase-contrast (*left*) or bright-field (*right*) illumination. Only lacZ-expressing cells (X-gal positive) with varying levels of expression (examples of intense X-gal stain, *; or modest X-gal staining, <) are visible under bright-field illumination (*right*, *top* and *bottom*). With longer X-gal incubations, a low level of endogenous galactosidase activity (X-gal staining) was detected in mock-infected cells.

after 14 days in culture (Table 2). To further determine the health of the progenitor cells, we performed a clonogenic assay. The number of colonies from fresh normal bone marrow was 73 ± 6.7/10^5 MNCs. The clonogenic capacity of the marrow cells decreases with time in culture to 42 and 31 at days 7 and 14, respectively (Table 3). There was no difference in the clonogenic capacity of G207-infected cells. Colonies in both mock and HSV-infected groups were dominated by CFU-granulocyte/monocyte.

Phenotypic Alterations of Human Bone Marrow Cells after HSV Infection. There was a significant change in the phenotype of cells present in the HSV-infected cultures at 14 days p.i., consistent with the proliferation of bone marrow cells. In particular, there was a significant increase in the monocyte population (CD14+; Table 2). The expression of CD38, a nonlineage specific marker of early or activated phenotypes (46), was also elevated in the infected cells. There was no significant difference in $CD3^+$ or $CD19^+$ cells after HSV infection (Table 2).

Susceptibility of Human Cancer Cells to HSV Cytotoxicity. As a model for stem cell transplantation purging, we tested two human breast cancer cell lines, MDA-MB-231 and MDA-MB-435, and a human leukemic cell line, K562. Among human breast cancer cell lines, MDA-MB-435 cells are very sensitive to G207 cytotoxicity (Fig. 3*A*), whereas MDA-MB-231 is poorly sensitive, with only about 40% of tumor cells killed at a MOI of 0.1, as determined by trypan blue dye exclusion (Fig. 3*B*). MDA-MB-231 is also less sensitive to killing by wild-type HSV-1 KOS. Similar results were reported previously (35). K562 cells are resistant to the cytopathic activity of HSV-1, even to wild-type KOS (Fig. 3*C*).

Purging of Breast Cancer Cells in Human Bone Marrow. Human breast cancer cells MDA-MB-435 and MDA-MB-231 were mixed with human bone marrow cells *in vitro* and infected with G207 at a MOI of 0.1. A colony-forming assay in methylcellulose was performed to detect contaminating tumor cells after infection. There was a significant decrease in the number of colonies (CFU-Tr) observed after G207 infection with either MDA-MB-231- or MDA-MB-435-contaminated bone marrow (Table 4). No MDA-MB-435 colonies were observed 6 days p.i., even with 10% contaminating tumor cells in the initial culture, whereas the number of CFU-Trs in mock-infected cultures doubled from days 3 to 6 (Table 4). Colony formation of MDA-MB-231 was inhibited by G207 infection, but a few colonies were still observed at day 6 p.i. in the 10% contaminating cell cultures (Table 4), indicative of the decreased susceptibility of these cells to G207 replication (Fig. 3*B*). There were no colonies formed when bone marrow cells alone were plated.

Table 3 *Clonogenic capacity of HSV-infected human bone marrow cells*

Bone marrow cells were infected with G207 or KOS at a MOI of 0.1, and the number of colonies (CFU-culture) from 10^5 MNCs was determined 7 and 14 days p.i. Values are the mean ± SE (n = 6). The number of colonies from fresh bone marrow (in the absence of *in vitro* culture) was 73 ± 7/10^5 MNCs after 2 weeks culture in semi-solid medium.

	Day 7	Day 14
Mock	42 ± 7	31 ± 5
G207	48 ± 7	29 ± 3
KOS	39 ± 4	27 ± 3

Table 4 *Effect of G207 infection of breast cancer cell-contaminated human bone marrow on CFU-Tr*

Human bone marrow cultures were contaminated with 1, 5, or 10% MDA-MB-435 (435) or MDA-MD-231 (231) breast cancer cells. Cells, as indicated, were mock-infected or infected with G207 at a MOI of 0.1. Three or 6 days after infection, CFU assays were initiated, and colonies (CFU-Tr) were counted 7 days later.

	Day 3			Day 6		
	1%	5%	10%	1%	5%	10%
435/Mock	14 ± 6	83 ± 3	163 ± 10	44 ± 3	146 ± 5	297 ± 8
435/G207	7 ± 1	42 ± 2	88 ± 7	0	0	0
231/G207	11 ± 2	62 ± 3	103 ± 9	0	0	16 ± 2

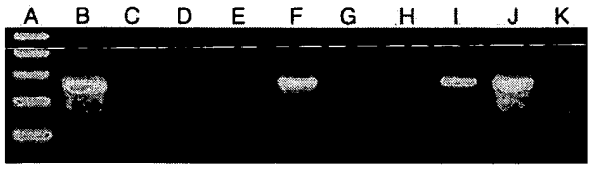

Fig. 4. RT-PCR detection of keratin-19. *Lane A*, 100-bp marker; *Lane B*, MDA-MB-435 cells alone; *Lanes C–E*, bone marrow contaminated with 1, 5, or 10% MDA-MB-435 cells, respectively, and treated with G207; *Lane F*, MDA-MB-231 cells alone; *Lanes G–I*, bone marrow contaminated with 1, 5, or 10% MDA-MB-231 cells, respectively, and treated with G207; *Lane J*, human skin; and *Lane K*, bone marrow alone. Total RNA was extracted from the cultures 6 days p.i. (pool of triplicate wells), reverse-transcribed, and amplified. The keratin-19-amplified fragment is 462 bp. This is a representative example from three separate experiments. The breast cancer cells and skin were positive, whereas the bone marrow cells were negative for keratin-19.

A more sensitive assay for contaminating tumor cells is RT-PCR. Keratin-19 mRNA is highly expressed in epithelial cells and most breast cancer cells and has been used to detect micrometastases of breast cancer in lymph nodes (47). RNA was extracted from G207- and mock-infected bone marrow cultures contaminated with breast cancer cells, and keratin-19 expression was characterized. Human bone marrow was negative for keratin-19 (Fig. 4*K*), and the human breast cancer cell lines were strongly positive (Fig. 4, *B* and *F*). At 6 days after G207 infection (MOI, 0.1), keratin-19 RNA was not detected in the MDA-MB-435-contaminated bone marrow (Fig. 4, *C–E*) but was detected in the 10% MDA-MB-231-contaminated bone marrow (Fig. 4*I*).

DISCUSSION

A concern in autologous stem cell transplantation is contamination of the graft with tumor cells. For patients with breast cancer, contamination of bone marrow or PBPCs occurs frequently, even in lymph

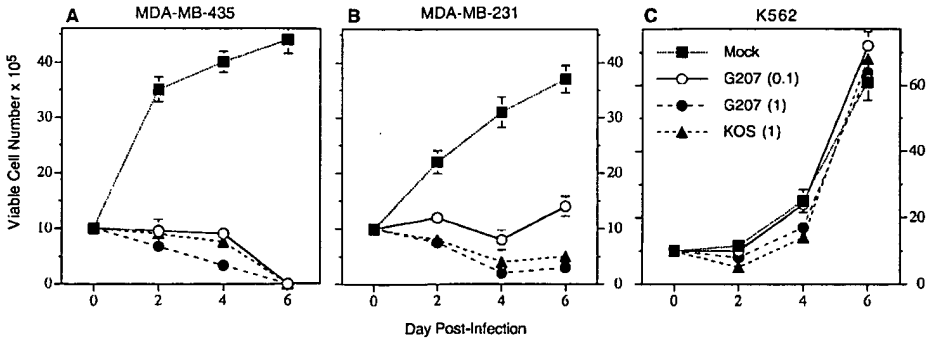

Fig. 3. Sensitivity of human tumor cells to HSV-1 cytotoxicity. Human breast cancer cell lines MDA-MB-435 (*A*) and MDA-MB-231 (*B*) and human chronic leukemia myeloid cell line K562 (*C*) were infected with G207 or KOS at the indicated MOIs (in parentheses). Values are the mean ± SE of triplicate samples.

node-negative patients (12, 48). The demonstration in gene marker studies of transduced bone marrow that tumor cells in relapsed patients contained the marker gene suggests that tumor cell contamination at transplant is associated with relapse (49, 50). We describe a novel approach to purging occult breast cancer cells from bone marrow in the context of autologous stem cell transplantation using an attenuated, replication-competent HSV-1 vector, G207. G207 was developed for the treatment of brain tumors (32), but it has a number of features that make it attractive for purging of bone marrow. It replicates in dividing cells, undergoing a lytic infection with consequent cell death, whereas replication in nondividing cells is restricted, targeting viral spread and cytotoxicity to tumor cells. Among its safety features are hypersensitivity to antiviral drugs such as acyclovir and ganciclovir, multiple mutations that make reversion negligible, limited reactivation from latency (51, 52), and, most importantly, lack of neuropathogenicity (38, 39). The HSV thymidine kinase gene has been used as a "suicide" gene after allogeneic bone marrow transplantation to delete transduced lymphocytes during graft-*versus*-host disease (53). G207 contains a functional thymidine kinase gene that would permit deletion of G207-infected bone marrow cells after transplantation.

The susceptibility of tumor cell lines to G207 replication and cytotoxicity can vary (33, 35). Among the human breast cancer cell lines we have tested, MDA-MB-435 is the most susceptible and MDA-MB-231 the least susceptible to G207 and wild-type strain F (35). As a model for *ex vivo* bone marrow purging, human bone marrow was mixed with varying amounts of human breast cancer cells. G207 was able to eliminate all of the detectable contaminating breast cancer cells, both MDA-MB-435 and MDA-MB-231, from human bone marrow at a rather low viral dose (MOI, 0.1), with the exception of MDA-MB-231 at the highest ratio (10%). This level of tumor cell contamination (10%) is much higher than would be seen in the clinic, where it is frequently $<0.01\%$ (17), and patients are usually excluded from transplant when they have tumor cell contamination $<1\%$. It is possible that all of the MDA-MB-231 cells might have been destroyed if the incubation period was continued for longer than 6 days or a higher MOI was used. Among other tumors that are treated with high-dose chemotherapy and autologous stem cell transplantation (54), human neuroblastoma cells are similarly sensitive to G207 *in vitro*.[4]

In vivo, in solid tumor models in nude mice, a single intratumoral inoculation of G207 can significantly inhibit tumor growth and cure established tumors (33, 35). However, s.c. or intracerebral tumor cell implants of MDA-MB-231 are not inhibited by G207, presumably because viral replication is not sufficient (35). In addition to the direct cytopathic effects of G207 on tumor cells, inoculation of syngeneic tumors in immune-competent mice induces a systemic antitumor immune response (36, 37). This immune response involves tumor cell specific $CD8^+$ CTLs and can inhibit the growth of noninoculated established tumors (36, 37). If such induction were to occur during bone marrow purging, this might facilitate elimination of residual or drug-resistant tumor cells remaining after high-dose chemotherapy. This also provides an incentive to combine this approach with post-transplantation immunotherapy, such as IL-2 infusions and others (55–57).

Human bone marrow cells were very resistant to HSV-1 replication and cytotoxicity, even to wild-type virus. However, they were efficiently transduced by dvHCL, suggesting that defective HSV vectors might be useful for gene therapy applications involving hematopoietic stem cells. Earlier studies showed that most human monocytes and lymphocytes are resistant to HSV infection *in vitro*, with replication blocked after adsorption but before expression of immediate-early proteins (58, 59). Culture of these cells *in vitro* and/or mitogen-stimulation led to increased susceptibility to HSV-1 replication (59–61). Interestingly, it was found that nonneuroinvasive strains of HSV-2 replicate poorly in stimulated human monocytes and that rescue of the neuroinvasive phenotype also rescued the ability to replicate in stimulated monocytes (62). G207 infection of human bone marrow cells resulted in a 2–3-fold increase in viable cells when compared with controls after 2 weeks in culture, even in the presence of growth factors. This could be attributable to the increased proportion of mitotically active cells. Alternatively or in addition, there might be decreased apoptosis, because infection with KOS had only a minimal effect on the number of viable cells, although the proportion of mitotically active cells increased. It has been reported that HSV-1 infection of peripheral blood MNCs induces apoptosis in a portion of the infected cells (63). The increased cell number could also be induced by conditioned media, indicating that G207 infection likely stimulated the secretion of soluble growth factors likely to be beneficial for the engraftment of progenitors after transplantation.

These studies suggest that attenuated replication-competent HSV-1 vectors such as G207 have potential for purging occult tumor cells from human bone marrow. No toxicity has been seen after i.v. injection of G207 in mice, athymic or immune-competent (38, 64). If infusion of G207 were a concern in this patient population, antiviral drugs such as acyclovir could be used. Expression of lacZ from the ICP6 viral promoter was detected in a small proportion of the G207-infected human bone marrow cells within the first few days p.i. It is possible that use of other promoter constructs might have led to expression in larger numbers of cells, as seen with dvHCL. The use of oncolytic viruses for tumor cell purging could be augmented by the expression of cytokines or immune-modulatory genes that would enhance the engraftment of stem cells or facilitate antitumor immune responses (65). Such genes could be recombined into the G207 backbone (*i.e.*, in place of lacZ) or defective vectors expressing such genes generated in combination with G207 (66).

[4] P. Hernaiz-Driever and S. Rabkin, unpublished data.

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Amendment Under 37 C.F.R. § 1.116
Expedited Procedure - Art Unit 1632

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 4747
Chiocca and Chung	Art Unit: 1632
Appl. No. 09/653,277	Examiner: J. Woitach
Filed: August 31, 2000	Atty. Docket: 0609.4880002/JAG/KRM
For: **Cell-Specific and/or Tumor-Specific Promoter Retargeting of Herpes Gamma 34.5 Gene Expression**	

Declaration of E. Antonio Chiocca Under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Mail Stop AF

Sir:

The undersigned, Dr. E. Antonio Chiocca, declares and states that:

1. I am a co-inventor of the above-captioned U.S. patent application.

2. I am an associate visiting neurosurgeon at the Massachusetts General Hospital (MGH) and associate professor of surgery at Harvard Medical School. In addition, I am the director of the Molecular Neuro-Oncology Laboratory (section of Neuro-Genetic Surgery) at MGH, where I conduct and supervise neurosurgical research in the fields of gene transfer, gene therapy, vector engineering, and neuro-genetic surgery.

3. I am the subject of the *Curriculum Vitae* attached as **Exhibit 1**. On the basis of the information and facts contained in this document, I submit that I am an expert in the fields of neurosurgery, neuro-genetic surgery, and neuro-oncology.

4. I have read and understand the Office Action dated May 20, 2003, Paper No. 17, particularly pages 5-10 in which the claims have been rejected under 35 U.S.C. §112, first paragraph, for non-enablement of "other types of neoplastic cells" (aside from CNS and perfuse metastatic liver and colon cancer) and "other promoters" (aside from B-myb).

5. Subsequent to the filing of the above-referenced patent application, I, together with other investigators, have obtained additional *in vitro* and *in vivo* experimental data, which demonstrate tumor-specific viral replication using (1) the nestin promoter; and (2) the prostate specific antigen (PSA) promoter, to drive expression of the herpes γ34.5 gene.

The details of the experiments conducted are described below. The Figures referenced below can be found in attached **Exhibit 2.**

6. To show that tumor-specific promoters can be used to drive expression of the γ34.5 gene of HSV1, thereby providing tumor-specific viral replication, we selected the promoter for the intermediate filament protein, nestin. Substantial amounts of nestin have been detected in human gliomas and glioblastomas and nestin immunostaining has frequently been observed in highly malignant gliomas, especially glioblastomas. In contrast, nestin is rarely detected by immunostaining in non-neoplastic brain tissues, occurring sometimes faintly in vascular endothelial cells. *See*, Dahlstrand, J., *et al.*, *Cancer Res. 52*: 5334-5341 (1992)(copy attached as **Exhibit 3**).

7. In Figure 1, schematics of the oncolytic HSV1s that we have generated by using the HSVQuik method (Saeki, Chiocca, et al. unpublished) are shown. rQNestin34.5 represents a new tumor-selective HSV1, in which both γ34.5 genes have been deleted, as well as the endogenous copy of ICP6. In the ICP6 locus, we added one copy of the γ34.5 gene under the control of the nestin promoter (composed of 714 base pairs of the nestin enhancer and the minimum promoter from Hsp78). This strategy is similar to the one used to generate Myb34.5, which was exemplified and described in the present application. As a control, wild-type HSV1 (rQHsv1) was also included.

8. To assess the selectivity in replicative ability of each mutant, a panel of human glioma cells, each expressing nestin to different levels were plated in a subconfluent fashion into dishes and, then, each mutant (rHSVQ1 and rQNestin34.5) were added at doses of 1×10^3 or 1×10^4 (Figure 2). After washing cells to remove uninfecting virus, replication was assayed by visual inspection of plaque size (Figures 3A-3D) and by titrating the production of progeny viruses, as well their cytotoxicity (Figures 4A-4B). The glioma cells employed were human U251 and U87ΔEGFR glioma cells, which express very high levels of nestin, and human T 98G and Gli36ΔEGFR, which express lower levels of it. Figures 3A and 3B represent plaque assays where the size of rHSVQ1 and rQNestin34.5 viral plaques (measured by green fluorescence) can be compared in the U251 (panel A) and U87ΔEGFR (panel B) cells. It shows that rQNestin34.5 produces larger plaques than the wild-type virus rHSVQ1. Figures 3C and 3D represent plaque assays where the size of of rHSVQ1 and rQNestin34.5 viral plaques (measured by green fluorescence) can be compared in the T98G (panel C) and Gli36ΔEGFR (panel D) cells. The size of plaques in this experiment shows that there is little qualitative difference between the 2 viruses.

9. The experiments described in paragraph 8 indicate that, in glioma cells expressing high levels of nestin, rQNestin34.5 spreads better than wild-type virus. This is possibly due to the fact that nestin promoter will drive γ34.5 gene expression in these cells to a much higher level than that driven by the wild-type, endogenous HSV1 prom ter. In glioma cells expressing low levels of nestin, spread of rQNestin34.5 is similar to that of wild-type rQHSV1, possibly because γ34.5 gene expression is similar in this case.

10. The above conclusion is further supported by the titration experiment where progeny virus production was assayed in all four cell lines (Figure 4) and by the cytotoxicity experiment where the number of surviving tumor cells was determined 3 days after infection. Figure 4A shows that, in the U251 high-nestin producing cell line, the production of rQNestin34.5 was 3 orders of magnitude larger than that of rQHSV1. Not surprisingly, the higher level of virus production correlated with higher levels of glioma cell killing. 80% of U251 cells survived 3 days after rHSVQ1 infection, while less than 5% of cells survived after rQNestin34.5 infection. Similar results were obtained when the U87ΔEGFR glioma line was used: In the viral titration assay, the number of generated progeny viruses was 2 orders of magnitude different for rQNestin34.5, when compared to wild-type rQHSV1. In the cytotoxicity assay, rHSVQ1 had little effect on survival at 3 days, while 10% of rQNestin34.5-infected cells survived.

11. When the cells that expressed relatively weaker levels of nestin (T98G and Gli36ΔEGFR) were used, both the virus replication assay and the cytotoxicity assays showed that rQNestin34.5 replicated in and killed these cells more efficiently than wild-type rQHSV1 (Figure 4B). Taken in conjunction, these experiments demonstrate that rQNestin34.5 will destroy glioma cells that express high levels of nestin better than wild-type virus, while in cells that express lower levels of nestin, rQNestin34.5 still works much better than wild-type HSV1.

12. Next, we determined whether rQNestin34.5 replicated in normal human astrocytes. Normal human astrocytes were plated on a dish and then wild-type rQHSV1 or rQNestin 34.5 were added (Figure 5). Four days later, the number of virus progeny generated was assayed. Figure 6 shows that astrocytes were infected with both viruses as measured by GFP fluorescence (Top panel). However, rQNestin34.5 replicated to the same level in astrocytes as wild-type rQHSV1. We then compared the amount of replication in each tumor cell line vs. astrocytes for each virus. Table 1 shows the ratio of replication in glioma cell line vs. astrocyte for rQNestin34.5 vs. wild-type rQHSV1.

Table 1 – Replication ratio in indicated tumor cells vs. astrocytes for viruses

	U251	U87ΔEGFR	T98G	Gli36ΔEGFR
RQNestin34.5	112.5	62.5	11.25	6.25
RQHSV1	0.1	0.1	1	0.8

In the case of rQNestin34.5, the ratio of replication in the high nestin producer cells (U251) was 900,000/8,000 = 112.5 and in U87ΔEGFR was 500,000/8,000 = 62.5. In the low nestin producer cells, it was 90,000/8,000 = 11.25 and 50,000/8,000 = 6.25. For wild-type rQHSV1, the ratio in the high nestin producers was 500/5,000 = 0.1 and 5,000/5,000 = 1 and in the low nestin producers it was 4,000/5,000 =0.8. Taken in conjunction, this study shows that rQNestin34.5 possesses a high therapeutic index for replication in glioma cells vs. astrocytes, when compared to wild-type rHSVQ1. In other words, rQNestin34.5

replicated 1000 times better in tumor vs. astrocytes than rQHSV1 in a high-nestin producer cell to 10 times better in a low-nestin producer.

13. We then grew U87ΔEGFR glioma cells into the subcutaneous flank of athymic mice and when tumors became palpable, injected them with Hanks' buffered saline solution (HBSS), wild-type rHSVQ1, rQNestin 34.5, or Myb34.5 (Figure 7). The tumor growth curves show that rQNestin34.5 suppressed tumor growth as well as Myb34.5 and more efficiently than rQHSV1. This shows that expressing the γ34.5 gene under control of the nestin glioma-specific promoter resulted in efficient tumor growth inhibition *in vivo*, similar to that demonstrated by Myb34.5.

14. To further prove that tumor-specific promoters can be used to drive expression of γ34.5, thus increasing selectivity of a virus for tumor cells, we constructed an HSV1 where the prostate-specific promoter for the prostate-specific antigen (PSA) was used to drive expression of γ34.5 (Figure 8). rQPSA34.5 thus employs the PSA promoter-enhancer element to drive expression of γ34.5. rHSVQ1 was previously described. In addition, we regenerated a virus (rQMyb34.5) that possesses genetic similarity to Myb34.5 in that the B-Myb promoter was used to drive expression of γ34.5. The major difference between Myb34.5 and rQMyb34.5 is that the former was generated by a homologous recombination method in mammalian cells, while the latter was generated by a site-specific recombination method in bacteria.

15. We first tested the spread of each virus in a plaque formation assay. Wild-type rHSVQ1, rQMyb34.5, and rQPSA34.5 were used to infect PSA-positive human LnCAP prostate cancer cells. Figure 9 shows that plaques formed by rQMyb34.5 and rQPSA34.5 were quantitatively larger than those formed by rHSVQ1, indicating that the PSA promoter drove expression of gamma34.5 and viral replication in these cells to a higher level than that obtained when endogenous HSV1 promoters were used. When PSA-negative human DU145 prostate cancer cells were used, the size of plaques produced by rQPSA34.5 was not qualitatively different than the size of plaques produced by rQHSV1 or rQMyb34.5 (Figure 10). In a viral replication and cytotoxicity assay, rQPSA34.5 generated more virus progeny and produced more cytotoxicity in PSA-positive LnCAP prostate cancer cells than wild-type rHSVQ1 (Figure 11). The production of virus progeny was quantitatively similar between rQMYb34.5 and rQNPSA34.5, indicating that both the B-Myb promoter and the PSA promoter worked well in this cell line. There was though evidence for increased cytotoxicity exhibited by rQPSA34.5 compared to that mediated by rQMyb34.5. In the PSA-negative DU145 cell line, rQMyb345. and rQPSA34.5 still replicated better, albeit by only one order of magnitude, than wild-type rQHSV1 showing that both the B-Myb and the PSA promoter were active. This was further confirmed in the cytotoxicity assay. Taken in conjunction, these results show that the PSA promoter can also be used to drive expression of the γ34.5 gene in prostate cancer cells.

16. I have read and understood 37 C.F.R. § 10.18 (b) and (c).

11/17/2003
Date

E. Antonio Chiocca, M.D., Ph.D.

SKGF_DC1:200243.1

Exhibit-1

Exhibit 1

CURRICULUM VITAE

Date prepared: 08/27/2003

PART I: General Information

NAME: E. Antonio Chiocca

OFFICE ADDRESS: Brain Tumor Center
Cox 315
Massachusetts General Hospital
Boston, MA 02114

HOME ADDRESS: 36 Crystal St.
Wakefield, MA 01880
Phone: 781-224-2850

DATE OF BIRTH: 11/15/1959

PLACE OF BIRTH: Padua, Italy

EDUCATION:

1982 B.S. University of Texas at El Paso.
1988 M.D. University of Texas Medical School at Houston.
1988 Ph.D. Graduate School of Biomedical Sciences, University of Texas Health Science Center, Houston.

POSTDOCTORAL TRAINING:

1988 - 1989 Intern in General Surgery, University of Texas Health Science Center at Houston
1989 - 1995 Resident in Neurological Surgery, Massachusetts General Hospital, Boston, MA.

LICENSURE AND CERTIFICATION:

1988-present Texas
1989-present Massachusetts
1996-2000 Board-eligible in Neurosurgery
2000-present Certified in Neurosurgery by the American Board of Neurological Surgery

ACADEMIC APPOINTMENTS:

1995-1996 Assistant in Neurosurgery, Massachusetts General Hospital
1995-1996 Instructor in Surgery, Harvard Medical School
1996- 1998 Assistant Professor in Surgery (Neurosurgery), Massachusetts General Hospital and Harvard Medical School
1998-present Associate Professor in Surgery (Neurosurgery), Massachusetts General Hospital and Harvard Medical School.

HOSPITAL APPOINTMENTS:

1995-1996 Assistant in Neurosurgery, Instructor in Surgery, Massachusetts General Hospital
1996-1998 Assistant Professor in Neurosurgery, Harvard Medical School, Massachusetts General Hospital
1996- 2000 Staff Neurosurgeon, Cambridge/ Somerville Hospital
1996-2000 Staff Neurosurgeon, Whidden Hospital
1997-1999 Staff Neurosurgeon, Melrose-Wakefield Hospital
1997-1998 Staff Neurosurgeon, Emerson Hospital
1998-present Associate Professor in Surgery (Neurosurgery), Harvard Medical School, Massachusetts General Hospital.
1998- present Associate Visiting Neurosurgeon, Massachusetts General Hospital.

MEMBERSHIPS IN PROFESSIONAL SOCIETIES:

1990-present Congress of Neurological Surgeons
1999-present Member of the Executive Committee of the American Association of Neurological Surgery/Congress of Neurological Surgery Joint Section on Tumors
1999-present American Society for Gene Therapy
2001-present American Association of Neurosurgery
2001-present International Society for Neurovirology
2001-present American Society for Microbiology
2001-present American Society for Experimental Neurotherapeutics

EDITORIAL BOARDS

Editorial board member of Neoplasia, Cancer Gene Therapy, Journal of Neurovirology.

" AD HOC" JOURNAL REVIEWER (1993-PRESENT)

Science, Nature, Nature Medicine, Nature Genetics, Nature Biotechnology, Cancer Research, Clinical Cancer Research, Journal of Virology, Journal of Neurovirology, Neoplasia, Gene Therapy, Cancer Gene Therapy, Human Gene Therapy, Molecular Pharmacology, Journal of Neuropathology and Experimental Neurology, Journal of Neuro-oncology, Neurosurgery, Neurosurgical Focus, Journal of Gene Medicine, Oncogene, Molecular Therapy, PNAS, Experimental Neurology, Life Sciences, Molecular Cancer Therapy, Journal of Clinical Investigation.

AWARDS AND HONORS:

1985	Alpha Omega Alpha
1988	The Alumni Association Senior Student Research Award (U. Texas Medical School)
1988	Walter G. Sterling Outstanding Student Award (U.Texas Health Science Center)
1992 - 1994	James B. Plunkett Fellow of American Brain Tumor Association
1994	Congress of Neurological Surgeons Resident Award
1994	American Academy of Neurological Surgeons Honorable Mention
2001	National Brain Tumor Foundation "Charles B. Wilson" Award
2002	National Brain Tumor Foundation"NABTT Award"

NATIONAL COMMITTEES/ ADVISORY BOARDS

1997	Invited participant NCI/CTEP Conference on HSV for tumor therapy, Rockville, MD
1998	Invited participant, Preuss Foundation Advisory Meeting on Immunotherapy for Brain Tumors.
1999-present	Member of Executive Council American Association of Neurological Surgeons/ Congress of Neurological Surgeons Joint Section on Tumors
1999	Member, Abstract Review Group, American Society for Gene Therapy Annual Meeting
1999	Member, Abstract Review Group, International HSV Workshop.
2000	Invited participant NCI/NINNDS Progress Review Group on Brain Tumors, Bethesda, MD.
2000	Member, Abstract Review Group, American Society for Gene Therapy Annual Meeting.
2000-present	Member, Scientific Review Board, National Gene Vector Laboratories
2001	Chair, Program Committee, Tumor Section, AANS/CNS
2001	Invited participant NINDS Conference on Gene Therapy for the CNS, Rockville, MD.
2001	Coordinating reviewer, Abstract Review Group, American Society for Gene Therapy Annual Meeting
2002	Member, Abstract Review Group, 14th International Conference on Brain Tumor Research and Therapy
2001-present	Member of Scientific Advisory Council, American Brain Tumor Association
2002-present	Member of "Molecular Genetics and Oncology" study section of American Cancer Society
2001-present	"Ad hoc" Consultant NINDS and NCI for Program Project Reviews (NCI-C and D) and for NINDS NSD-B Study Section
2002-present	Member of Extra-Mural Subspecialty Writing Committee, ABNS.
2003	Member of Vector Committee, European Society for Gene Therapy.
2003	Member of Nominating Committee, American Society of Gene Therapy
2003	Chair, Program Committee, Tumor Section, AANS 2004.

BIOTECHNOLOGY CONSULTANTSHIPS

Oxford Biomedica, Millenium Pharmaceuticals, Prestwick Scientific Capital, Shanghai Biotech.

Part II:

A. Report of research

1. MAJOR RESEARCH INTERESTS:

Biologic therapies and applications to disorders of the central nervous system/ Functional genomics in brain tumors/ Immune responses to gene delivery vehicles/

2. NARRATIVE DESCRIPTION OF RESEARCH:

Biologic advances increasingly appear to provide a major technological advance in the treatment of disorders of the central nervous system (CNS). Stem cell biology, interactions between viral and tumor cell life cycles, definition of gene expression profiles in tumors represent fields of research that appear to provide possibilities for therapy. Currently active research areas include: 1) engineering more efficient "tumor-killing" vectors, 2) Finding and testing novel prodrug/ gene therapy strategies, 3) Defining more selective oncolytic viruses, 4) Characterizing the ability of bone marrow stem cells to "transdifferentiate" into tumor blood vessels or neurons in response to environmental stimuli, 5) Defining the role of the immune response in vector delivery of therapeutic molecules in tumors and in animal models of cerebral ischemia, 6) Developing models of gene transfer to ameliorate cerebrovascular ischemia, 7) Functional genomic studies using our iBAC system to study neurodegenerative disease.

3. RESEARCH FUNDING INFORMATION (ACTIVE GRANTS in BOLD) (GRANT FUNDING IN 2003 EXCEEDS $ 1,000,000 in DIRECT COSTS):

Year	Source	Role	Title
1992-1995	NIH-NINDS P01	Principal Investigator on sub-project 2	"Genetic alteration of central nervous system tumors"
1992-1994	AmericanBrain Tumor Assn.	Fellow	"Development of viral vectors"
1994-1995	"Elsa B. Pardee Foundation"	Co-Principal Investigator	A novel gene therapy for brain tumors"

1995-1996	"Oliver and Jenny Donaldson Charitable Trust"	Principal Investigator	"A novel gene therapy for brain tumors"
1996-2000	NIH-NCI P01	Principal Investigator on project 2	"Therapy of experimental brain tumors with prodrug-activating genes"
1997-1999	American Brain Tumor Association	Mentor to Dr. Chung	
2000-2005	**NIH-NCI P01 (1,000,000$ Direct for project 1)**	**Co-director of PPG and Principal Investigator on project 1**	**"Pharmacologic enhancement of viral oncolysis"**
1999-2000	Schering-Plough Sponsored Research	Principal Investigator	"Therapy of brain tumors with SCH5800 and PTEN adenoviruses"
1999-2004	**NIH-NCI R01**	**Collaborator (20%)**	**"Herpes simplex virus for hepatic metastases"**
2000-2005	**NIH-R01**	**Collaborator (20%)**	**"Imaging gene transfer by transferrin receptor expression"**
1999-2001	NIH-R21	Principal Investigator	"Interdisciplinary modeling of tumor complexity"
1999-2001	American Brain Tumor Asscoiation	Mentor to Dr. Smith	
2000-2203	The Wellcome Trust Foundation Travelling Fellowship	Mentor to Dr. Wade-Martins	
2000-present	**The Carolyn Frye Halloran Fund for Brain tumor Research**	**Principal Investigator (Sundry Fund)**	**"Brain tumor research at MGH"**
2000-present	**The Berkowitz-Knott Fund for Brain Tumor Research**	**(Sundry Fund)**	**"Brain tumor research at MGH"**
2001-2003	**The Cleveland Clinic Foundation Award "Finding a cure for glioblastoma"**	**Principal Investigator**	**"Oncolytic virus and immunosuppression in subhuman primates: preclinical toxicity studies"**
2001-2002	Prestwick Scientific Capital Sponsored Research	Principal Investigator	"Oncolytic HSV for prostate cancer"

2002-2004	**American Brain Tumor Association**	**Mentor to Dr. Fulci**	
2002-2005	**NIH-NINDS R01 (750K direct)**	**Principal Investigator**	**"HSV amplicons for cerebral ischemia"**
2001-2002	National Brain Tumor Foundation (C.B. Wilson Award)	Principal Investigator	"Stem cells for brain tumors"
2002-2003	**Accelerate Brain Cancer Cure, Inc.**	**Principal Investigator**	**"Intra-arterial OV and immunosuppression in subhuman primates"**
2002-2004	**Harvard Center for Neuroregeneration and Repair**	**Principal Investigator**	**Biology of Tau-associated dementias studied with HSV amplicons**
2002-2005	**NIH R21**	**Co-Principal Investigator**	**"HSV amplicons and Stem cells "**
2002-2003	**National Brain Tumor Foundation (NABTT Award)**	**Principal Investigator**	**"Stem cells for brain tumors"**
2003-2008	**NIH-BRP-R01 (3,200,000 direct)**	**Principal Investigator**	**"Interdisciplinary Tumor complexity models"**
PENDING			
2002	NIH NINDS	PI (Score: 36% at first submission)	" Tau-associated dementias: iBAC study of alterantive splicing
2002	NIH-NINDS R01	Principal Investigator (Last Score: 32 percentile at second submission)	"Identification of promoters expressed in gliomas"

CURRENT FUNDING directly to Laboratory (as of August 2003)

NIH/NCI (Hochberg) 08/1/01-7/31/06
2P01CA69246-07
Gene Therapy of Brain Tumors (Project 1 – E.A. Chiocca)
Co-Principal Investigator
Goal: Engineering HSV-based viral vectors to target malignant brain tumors including a pre-clinical and clinical phase.
Annual directs: $ 226,085 Indirects: $ 165,042

NIH/NCI (Hochberg) 08/1/01-7/31/06
2P01CA69246-07
Gene Therapy of Brain Tumors (Core B – Hochberg)
Co-Principal Investigator
Goal: Engineering HSV-based viral vectors to target malignant brain tumors including a pre-clinical and

clinical phase.
Annual directs: $ 134,312 Indirects: $98,048

NIH (Hochberg) 03/1/98-12/31/03
5U01CA62406-09-S1
New Approaches to Brain Tumor Therapy Consortium
Collaborator
Goal: clinical protocols for glioblastoma treatment.

The Cleveland Clinic Foundation (Chiocca) 9/10/01-9/9/03
Finding the Cures for Glioblastoma Award
Principal Investigator
Goal: 1) To determine if oncolytic virus (OV) injuection by neuro-interventioanl intracerebral catherization of the arterial circulation of non-human primates is toxic. 2) To determine if the immunosuppressive agent, Cyclophosphamide, increases toxicity of intra-arterial OV.
Annual directs: $ 100,000

NIH (Tanabe) 1/7/00-12/31/03
5 R01 CA076183-03
Targeting Diffuse Liver Metastases with Herpes Virus
Co-Investigator
Goal: Show uptake of HSV vectors in diffuse liver metastases.

NIH (Chiocca) 4/1/02-3/31/05
1R01NS41571-02
HSV Amplicon Vectors for Cerebral Ischemia
Principal Investigator
Goal: To employ an intra-arterially administered HSV amplicon vector for the delivery of the nurine endothelial nitric oxide synthase (eNOS) cDNA into the cerebral endothelial cells of mice exposed to an ischemic insult.
Annual directs: $ 237,500 Indirects: $ 156, 038

Harvard Center for Neurodegeneration & Repair (Chiocca) 5/1/02-4/30/04
Core C
Validation of the iBAC system to study the Neurological Significance of Alternative Splicing
Co-Principal Investigator
Goal: Development of a novel high-capacity genomic DNA expression system to investigate the neuropathological Significance of alternative splicing: the tau paradigm.
Annual directs: $ 100,000

NIH (Saeki) 9/15/02-8/31/04
Genetic Engineering of Neural Stem Cells using HSV Amplicon Vectors
Co-Investigator
Goal: 1) Verify episomal replication and maintenance of HSV/EBV/genomic vectors and evaluate functional transgene expression from the vectors in dividing NSCs in culture, and 2) verify stable and cell-type-specific expression of genomic transgenes in terminally differentiated neurons and glial cells both *in vitro* and *in vivo*.
Annual directs: $ 118,750 Indriects: $ 82,490

NIH (Chiocca) 8/1/03-7/30/08
1 R01 CA85139-01A1
Interdisciplinary Tumor Complexity Modeling
Principal Investigator
Goal: 1) To develop a novel 3D in vitro assay system, suitable of displaying several key-features of multicellular tumor spheroids (MTS) in parallel over a prolonged period of time, and 2) To develop a set of related, innovative computational models to simulate brain tumor proliferation, generic and epigenetic heterogeneity, angiogenisis and most importantly, tissue invasion.
Annual Directs: $ 876,163 Indirects: $ 235,734

B. SELF REPORT OF TEACHING

1. Local

a. Medical/Graduate School courses

1996 -Genetics 208: Gene Therapy
-Lecturer
- Average number of students (undergraduate, graduate, postgraduate): 30
- Two hour preparation and one hour lecture

1997 -Same as above

1998- Harvard BBS Workshop: "Gene Therapy"
- Lecturer
- Average number of students (graduate): 20
- Two hour preparationa nd one hour lecture

b. Hospital courses and invited teaching presentations

1995 , 1996, and 1997 -Neurosurgical Grand Rounds
- Lecturer
- Average number (CME, postgraduates, residents, interns): 50
- Five hour prepararation and two one hour lectures

1994 - Neurology Grand Rounds
- Lecturer
-Average number (CME, Postgraduates, residents, interns): 100
- Two hour preparation and one hour lecture

1997 -Same as above

1996 -Boston University Combined Neurosciences Grand Rounds
-Lecturer
-Average Number: 30
-Two hour preparation and one hour lecture

1997 -Hematology/Oncology Grand Rounds
-Lecturer
-Average Number: 50
-Two hour preparation and one hour lecture

1997 -Medical Grand Rounds (Brigham and Womens' Hospital)
-Lecturer

-Average Number: 30
-One hour preparation and one hour lecture

1997 -Boston University Neurosciences/ Neurology Grand Rounds
-Lecturer
-Average Number: 30
-One hour preparation and one hour lecture

1998 -Neurosurgery Grand Rounds (Brigham and Womens'Hospital)
-Lecturer
- Average number (CME, postgraduates, residents, interns): 30
- One hour prepararation and one hour lecture

c. Advisory and supervisory responsibilities

Research:

1992 - 1995	Ming X. Wei, Ph.D., Post-Doctoral Fellow	
1993	Jeremiah S. Scharf, M.D. Ph.D. Candidate, Harvard Medical School	
1993 - 1994	Efstathios J. Boviatsis, M.D. Ph.D. , Neurosurgical Research Fellow	
1993 - 1994	Takashi Tamiya, M.D. Ph.D., Neurosurgical Research Fellow	
1994 - 1995	Yasuhiro Ono, M.D., Neurosurgical Research Fellow	
1996- 1999	Keiro Ikeda, M.D. Neurosurgical Research Fellow	
1996- 1999	Xiaoqun Jiang, M.D. Postdoctoral Fellow	
1996- 1999	Yoshinaga Saeki, M.D. Ph.D. Postdoctoral Fellow	
1996	Massimo Nicolo, M.D. Postdoctoral Fellow	
1997	Nuzhat Husain, MD Research Fellow	
1997-1999	Richard Chung, MD PhD	Neurosurgical Research Fellow
1997-2002	Thomas Deisboeck MD PhD	Postdoctoral Fellow
1997-2001	Tomotsogu Ichigawa, MD	Neurosurgical Research Fellow
1999-2001	Edward Smith, MD	Neurosurgical Research Fellow
1999-2002	Tatsuya Abe, MD	Neurosurgical Research Fellow
1999-2002	Hiroaki Wakimoto, MD PhD	Neurosurgical Research Fellow
2001	Manish Aghi, MD PhD,	Neurosurgical Research Fellow
2001	Moksa Ranasinghe	Student
2000-present	Richard Wade-Martins, PhD	Postdoctoral Fellow/Instructor
2000-present	Yoshinaga Saeki, MD PhD	Instructor
2000-present	Kinya Terada, MD PhD	Postdoctoral fellow
2001-present	Giulia Fulci, PhD	Postdoctoral fellow
2001-2002	Kaveh Asadi	Student
2002-present	Ryo Inouye, MD PhD	Postdoctoral fellow
2002-present	Shinji Yamamoto, MD PhD	Postdoctoral fellow
2002-present	Davide Gianni	Graduate Student
2002	Giuseppe Mirone	Medical Student
2002-present	Sean Lawler, PhD	Instructor

2002-present	Khalid Abbed, MD	Neurosurgical Research Fellow
2003-present	Therese Visted, MD PhD`	Postdoctoral Fellow
2003-present	Masayuki Nitta, MD PhD	Postdoctoral Fellow
2003	Martine Lamfers, PhD	Visiting Scientist
2003	Elizabeth Fontana	Summer Research Medical Student

Clinical Residents:

1995	Gordon Tang, M.D., Neurosurgical Resident
1995	Emad Eskandar, M.D. Neurosurgical Resident
1995	Richard Chung, M.D. Ph.D. Neurosurgical Resident
1995	Six general surgery interns rotating through the Massachusetts General Hospital Neurosurgery service
1995	Four Neurology residents rotating through the Massachusetts General Hospital Neurosurgery Service
1996-present	Neurosurgery Service residents

Awards/scholarships won by students, fellows in Dr. Chiocca's laboratory in last 3 years

- Dr. Edward Smith – Synthes Award, Mayfield Award, Resident Research Award Tumor Section (AANS, CNS), American Brain Tumor Association Fellowship
- Dr. Richard Wade-Martins – ASGT traveling fellowship award, Wellcome Trust Travelling Fellow Award
- Dr. Martine Lamfers – Travelling Fellowship Award (Dutch Cancer Society)
- Elizabeth Fontana – American Academy of Neurology Summer Research Medical Student Award
- Dr. Therese Visted – Travelling Fellowship Award (Norwegian Cancer Society)
- Dr. Khalid Abbed – American Brain Tumor Association (2-year research fellowship)
- Dr. Davide Gianni – Travelling Scholarship – Italian Association for Cancer Research
- Dr. Hiroaki Wakimoto – Uehara Medical Foundation Grant
- Dr. Richard Chung – American Brain Tumor Association Fellowship (2 year)
- Dr. Giulia Fulci – American Barin Tumro Foundation Fellowship (2-year)
- Dr. Yoshi Saeki – NINDS R21, ALS Association research grant

2. Invited National and International Lectures

May 1992	One hour platform presentation at University of Cincinnati Medical Center "Neurofest 1992"
July 1993	One hour platform presentation at Sixth Annual Conference on Immunology and Gene Therapy, Osaka, Japan
November 1993	Twenty minute presentation at Society for Neuroscience Annual Meeting, Washington, D.C.

May 1994 Twenty minute presentation at the American Brain Tumor Association Annual Meeting, Chicago, IL

October 1994 Resident Award Presentation at the Congress of Neurological Surgeons, Chicago, IL

November 1994 Two hour lecture at the Review Update Course for Neurologists and Neurosurgeons, Woods Hole, MA

June 1995 One hour lecture at the Canadian Congress of Neurologists and Neurosurgeons, Basic Science Review Course, Victoria, B.C., Canada

May 1995 One hour Neuroimmunology Seminar, The Ohio State University Medical School, Columbus, OH

May 1995 American Academy Of Neurology, Platform Presentation, Seattle, WA

April 1995 The Stanley Cobb Assembly of Neurology and Psychiatry, Platform presentation

November 1995 Twenty minute presentation, International Conference on Brain Tumor Research and Therapy, Silverado, CA

April 1996 Boston University Combined Neurosciences Grand Rounds

June 1996 Invited Speaker: "Gene Therapy of Hereditary Diseases and Tumors", Padova, Italy.

February 1997 Invited Speaker: "Third International Workshop in Genetics in Hematology-Oncology, Faculty of Medicine, Dusseldorf, Germany.

March 1997 Invited Speaker: Advances in Medicine, Sarasota Memorial Hospital, FL.

September 1997 Invited Speaker First International Workshop in Gene Therapy and Brain Tumors, S. Giovanni Rotondo (FG), Italy.

September 1997 Invited Speaker: XIIth International Conference on Brain Tumor Research and Therapy, Oxford, England.

September 1997 Invited Speaker (Note: Did not accept) XXV Annual Congress of the International Society for Pediatric Neurosurgery, Verona, Italy.

September 1997 Invited Panel member: Workshop on NF2, Present and Future, sponsored by the House Ear Institute and the National Neurofibromatosis Foundation, Rockville, MD

October 1997 Invited Speaker: Conference on Genetics and Cancer, Seacoast Cancer Center

and Novartis, Dover (NH).

February 1998 Invited participant: Preuss Foundation Symposium on Vaccine therapies for gliomas La Jolla (CA).

March 1998 Invited Speaker: University of Virginia Cancer Center Seminars on Gene Therapy, Charlottesville (VA).

March 1998 Invited Session Moderator: Biology of the Cerebral Vasculature '98, Salishna Lodge (OR).

April 1998 Invited Speaker: Symposium on Novel Cancer Treatments, University of Texas, El Paso (TX).

April 1998 Invited Speaker: Third Biennial Satellite Symposium on Brain Tumors, AANS/CNS Section on Tumors, Philadelphia, PA.

May 1998 Invited Speaker: Seminar, Clinical Gene Therapy Branch, National Institutes of Health, Bethesda, MD

June 1998 Invited Speaker: Seminar, Istituto Europeo Oncologico, Milan, Italy.

June 1998 Invited Speaker: 49th Annual Meeting of the German Society of Neurosurgery, Hanover, Germany.

July 1998 Invited Speaker: 12th International Symposium on Microsomes and Drug Oxidations, Montpellier, France.

September 1998 Invited Speaker: 13th International Conference of the European Society for Functional and Stereotactic Surgery, Freiburg, Germany.

September 1998 Invited Speaker: German Society of Neurosurgery, Hanover, Germany

October 1998 Invited Speaker: 48th Annual Meeting of the Congress of Neurological Surgeons, Seattle, WA.

October 1998 Invited Speaker: Second Conference on Cellular and Molecular Treatments of Neurological Diseases, Cambridge, MA.

October 1998 Invited Speaker: Workshop (The ALS Association), Philadelphia, PA.

November 1998 Invited Speaker: Seventh International Conference on Gene Therapy of Cancer, San Diego, CA

February 1999 Invited Speaker: MD/PhD Program Seminar, MD Anderson Hospital and Cancer Center, Houston, TX.

June 1999 Invited Speaker: Gordon Research Conference on Neurovirology, Concord, NH

June 1999 Invited Speaker: American Society for Gene Therapy, 2nd Annual Meeting, Washington DC.

December 1999 Invited Speaker: Department of Microbiology and Virology, Mt. Sinai School of Medicine, New York

February 2000 Invited Speaker: Department of Neurosurgery Grand Rounds, Memorial Sloan-Kettering, New York.

February 2000 Invited Speaker: 25th International Stroke Conference, American Stroke Association, New Orleans, LA

February 2000 Invited Speaker: Gordon Research Conference of Drug Development and Delivery, Ventura, CA

June 2000 Invited Speaker: Eurocancer 2000, Paris, France

September 2000 Invited Speaker: 4th International Conference on Cancer Treatment: Naples, Italy

October 2000 Invited Speaker: St. Jude Hospital Children's Center: Memphis, TN.

November 2000 Invited Participant. NINDS Conference on "Gene therapy for CNS disorders" Bethesda, MD

February 2001 Invited Grand Round Speaker, Boston University Department of Neurology: "Peripheral Nerve Injury"

April 2001 Invited Speaker: Cerebrovascular Biology 2001 (Triennial International Meeting), Cambridge, UK

April 2001 Invited Speaker: American Association of Neurological Surgeons Annual Meeting (Symposium on novel biologic therapies for gliomas), Toronto, Canada

May 2001 Invited Speaker: Society of Neurosurgery, Cleveland, OH

June 2001 Invited Speaker: Cleveland Clinic Symposium "Finding a cure for glioblastoma"

July 2001 Visiting Professor; Johns Hopkins Neurosurgery Grand Rounds

July 2001 Invited Speaker; Cornell Medical School Neurosurgery Grand Rounds

October 2001 Invited Speaker: International Conference on Gene and Cell Therapy, Parma, Italy.

October 2001 Invited Speaker: Annual Meeting of the Japanese Society of Neurosurgeons, Okayama, Japan.

November 2001 Invited Speaker: "Meet the Expert Session" Annual Meeting of the Society for Neuro-Oncology, Washington, DC.

January 2002 Visiting professor: Cleveland Clinic Neurosurgery Grand Rounds

February 2002 Invited Speaker: Cleveland Clinic Neuro-oncology Course, Naples, FL

June 2002 Invited Speaker: Fourth International Congress on Neurovirology, Dusseldorf, Germany.

September 2002 Invited Speaker Institute Besta, Milan, Italy

September 2002 Invited Speaker Europan Association of NeuroOncology (5th annual meeting) Florence, Italy

September 2002 Invited Speaker Sixth Detroit Symposium on Neurosurgery, Wayne State University

September 2002 Invited Speaker Curtis-Hunt symposium in Translational Neurosciences, Ohio State University

October 2002 Invited Speaker; Cleveland Clinic Blood-brain-barrier symposium

November 2002 Invited Speaker, American Association of Pharmaceutical Scientists Annual Meeting, Toronto.

March 2003, Invited Participant Italian Ministry of Health, Scientific Conference

March 2003, Invited Speaker "Transfer of vectors and cells across the blood-brain-barrier" Portland, OR

March 2003, Invited Speaker "International Conference on Oncolytic viruses, Banff, Canada.

May 2003, Invited Speaker, MD Anderson Hospital Monthly Speaker Series: "Oncolytic viruses"

June 2003, Invited Speaker, 2003 Annual Meeting of American Society for Gene Therapy: "Innate responses to HSV vectors"

July 2003, Invited Speaker, Cleveland Clinic Symposium "Finding a cure for glioblastoma"

July 2003, Invited Speaker, American Brain Tumor Association Annual Meeting for Barin

Tumor Patients/Families

September 2003, Organizer Of Third Annual Carolyn Frye-Halloran Symposium in Neuro-oncology, MGH.

Fall 2003, Organizer, Monthly MGH Lecture Series in Neuro-oncology (Invited Speakers: Drs. W. Cavenee, D. Bigner, Dr. R. DePinho)

October 2003, Invited Speaker, Invitrogen Conference in Genomics and Proteomics (San Diego, CA): "Studying functional genomics with the iBAC system"

November 2003, Invited Speaker, University of Pennsylvania Gene Therapy Unit "Host responses against oncolytic viruses"

November 2003, Invited Speaker, "Meet the expert session" Annual Meeting of Society fro Neuro-oncology (November 2003)

December 2003, Invited Speaker, Twelfth Annual Meeting of Cancer Gene Therapy (San Diego, CA)

c. SHORT REPORT OF CLINICAL ACTIVITIES:

1. Description of clinical practice.

The major areas of clinical practice are in the field of neurosurgery and involve patients with:

a) benign and malignant tumors of the central and peripheral nervous system,
b) complex spinal disorders, and
c) patients with peripheral nerve entrapment syndromes.

The office practice is in the Cox Building within the Massachusetts General Hospital. Patients are seen within this office setting, in the emergency room of the hospital and as an inpatient consultant. Furthermore, neurosurgical consultation is provided to requesting physicians at outside hospitals.Neurosurgical operative procedures are performed at the Massachusetts General Hospital.

2. Patient load.

Patients are seen every Monday and weekly averages include approximately 10 new and 10 followup patients. Operative procedures occur on Tuesdays, Thursdays and Fridays and number approximately 5 per week (average). Approximately 3-4 patients per day are on my service. For instance, the number of operative cases performed in 2000 was approximately 200.

3. Clinical contributions

I have participated and I am preparing novel experimental protocols for the use of gene transfer vectors against malignant gliomas. These include:

1. Harsh, G.R., **Chiocca, E.A.,** Hochberg, F.: Assessment of retroviral-mediated incorporation of HSV Thymidine kinase and ganciclovir in human malignant gliomas. Phase I trial for recurrent glioblastoma multiforme. Massachusetts General Hospital and Somatix Therapy Inc.

2. Dranoff, G., Soiffer, R., Hodi, S.F., Rhuda, C., Duda, C., Singer, S., Mentzer, S., Webb, I., Neuberg, D., Jung, K., Haluska, F., Lynch, T.J., Mihm, M., Tanabe, K., Sober, A., GAdd, M., Souba, W., Cosimi, A.B., **Chiocca, E.A.,** Wain, J.C.: A phase I study of vaccination with autologous, lethally irradiated melanoma cells engineered by adenoviral mediated gene transfer to secrete human granulocyte-macrophage colony stimulating factor. Dana Farber Cancer Institute and Massachusetts General Hospital.

3. **Chiocca, E.A.,** Barker, F.G.: A phase I, open-label, dose- escalation, trial of intratumoral injection with an E1B-attenuated adenovirus (active and UV-inactivated), ONYX-015, into recurrent malignant gliomas. A protocol of the NABTT CNS consortium. *Manuscript in preparation.*

4. A phase I, multi-institutional, open-label Study of BG-00001, a replication-defective adenvorius expressing human interferon-beta for recurrent malignant glioma. (E.A. Chiocca, MD PhD, MGH PI)

ORIGINAL REPORTS.

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7. Two other patents pending

Exhibit-2

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Figure 1

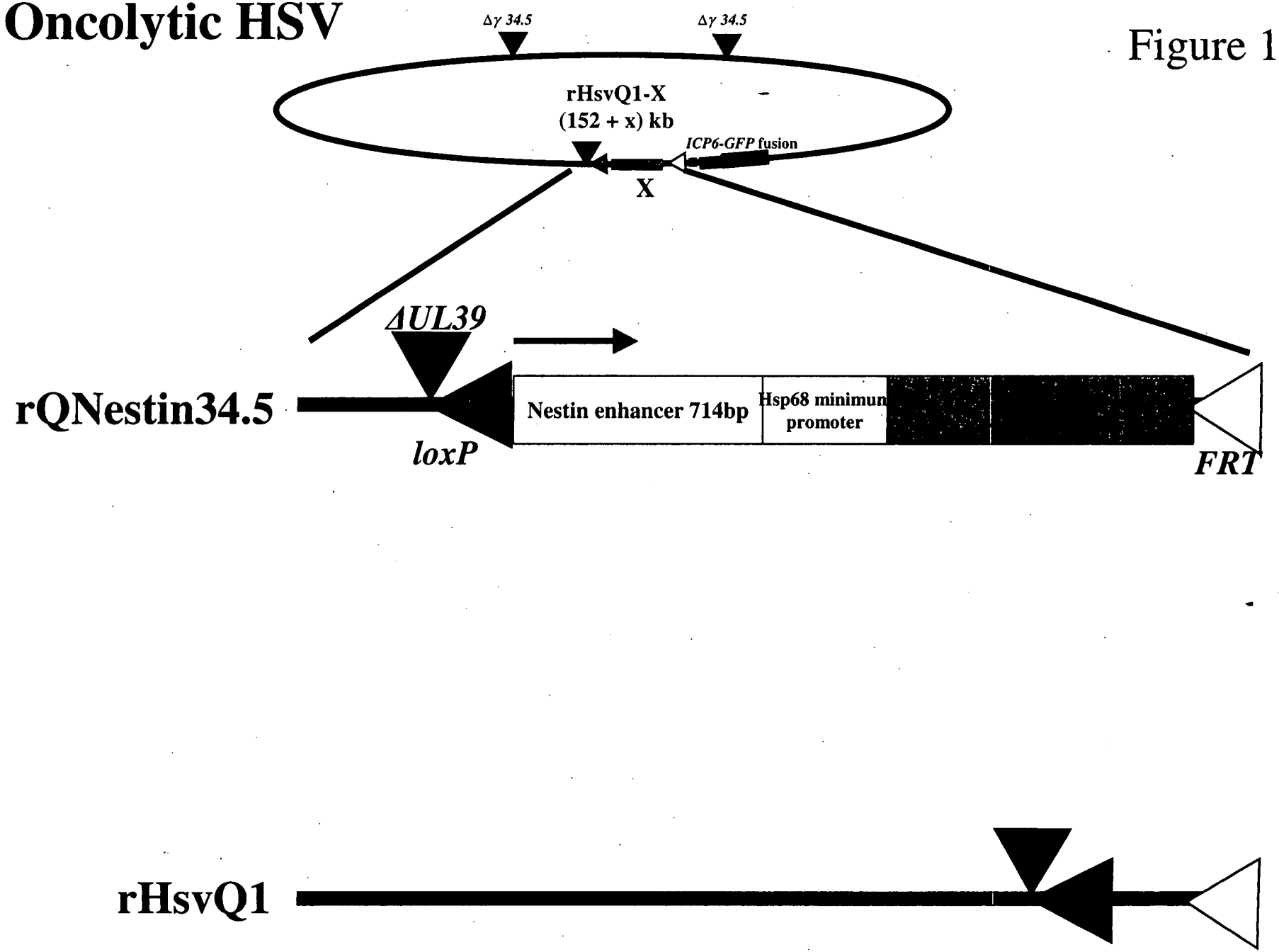

Oncolytic HSV
Δγ 34.5
Δγ 34.5
rHsvQ1-X
(152 + x) kb
ICP6-GFP fusion
X
ΔUL39
rQNestin34.5
Nestin enhancer 714bp
Hsp68 minimun promoter
loxP
FRT
rHsvQ1

Figure 2

Virus replication assay

Day 0
Infection

rHsvQ1
1.0×10^4 pfu or
1.0×10^3 pfu

6 well plate
subconfluent

rQNestin34.5
1.0×10^4 pfu or
1.0×10^3 pfu

6 well plate
subconfluent

↓

Wash with glycine-saline solution (pH 3) for removal of viruses remaining outside the cells 3 hours after infection

↓

Harvest cells and supernatant on day 3

↓

Titration

U251 (high nestin) infected with $1x10^3$ pfus

Fig. 3A

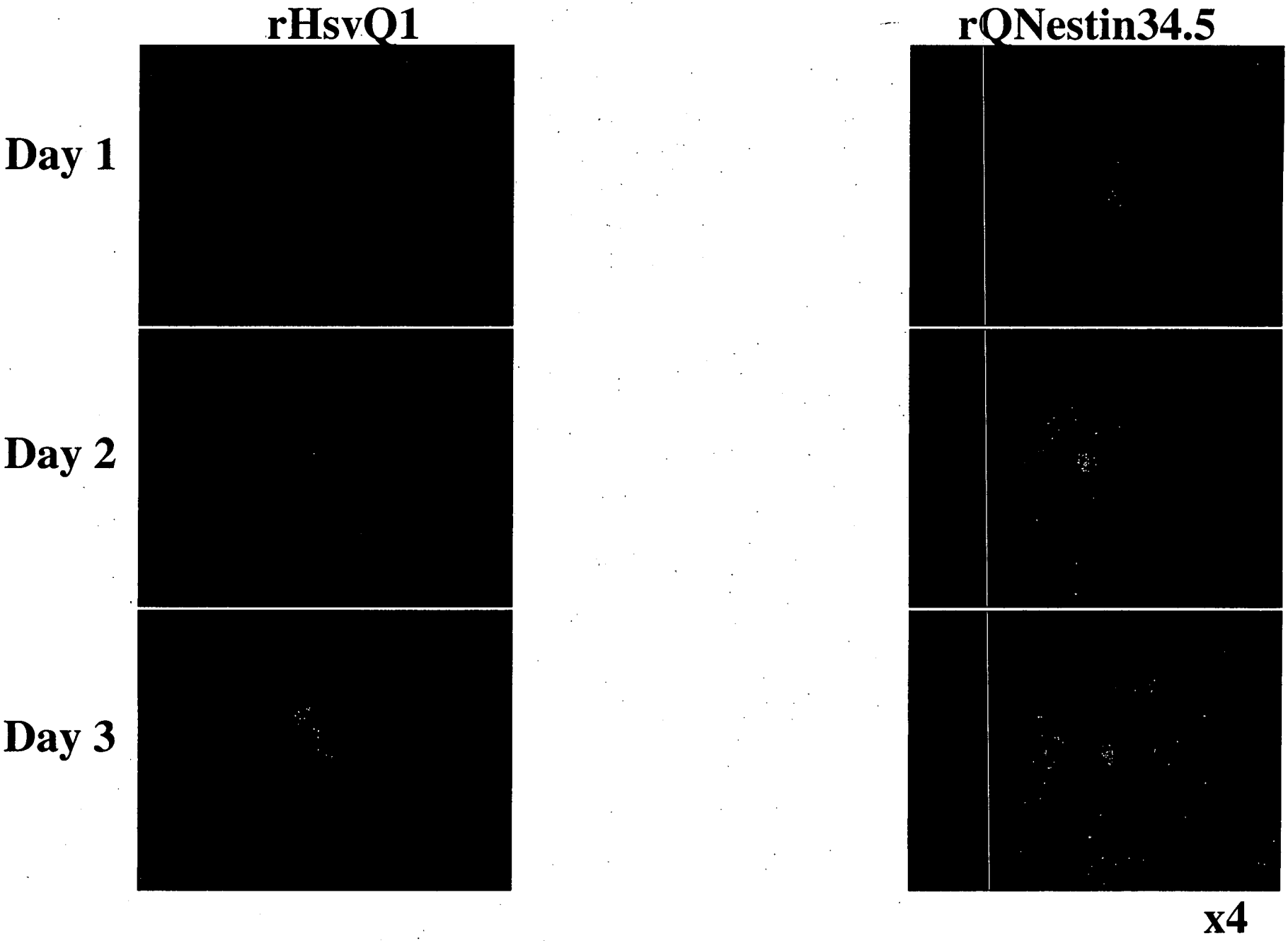


Fig. 3B

U87ΔEGFR (high nestin) infected with $1x10^4$ pfus

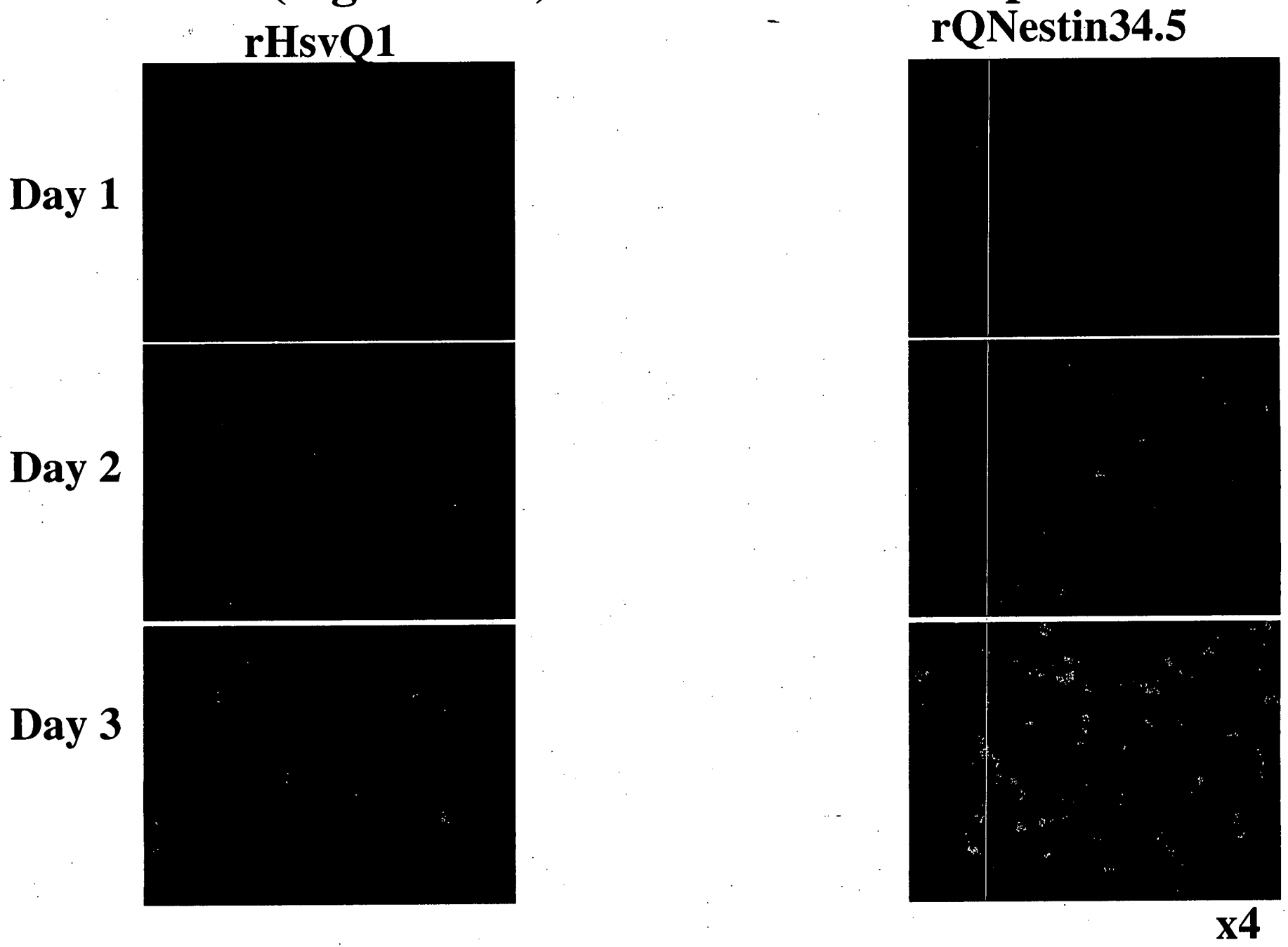
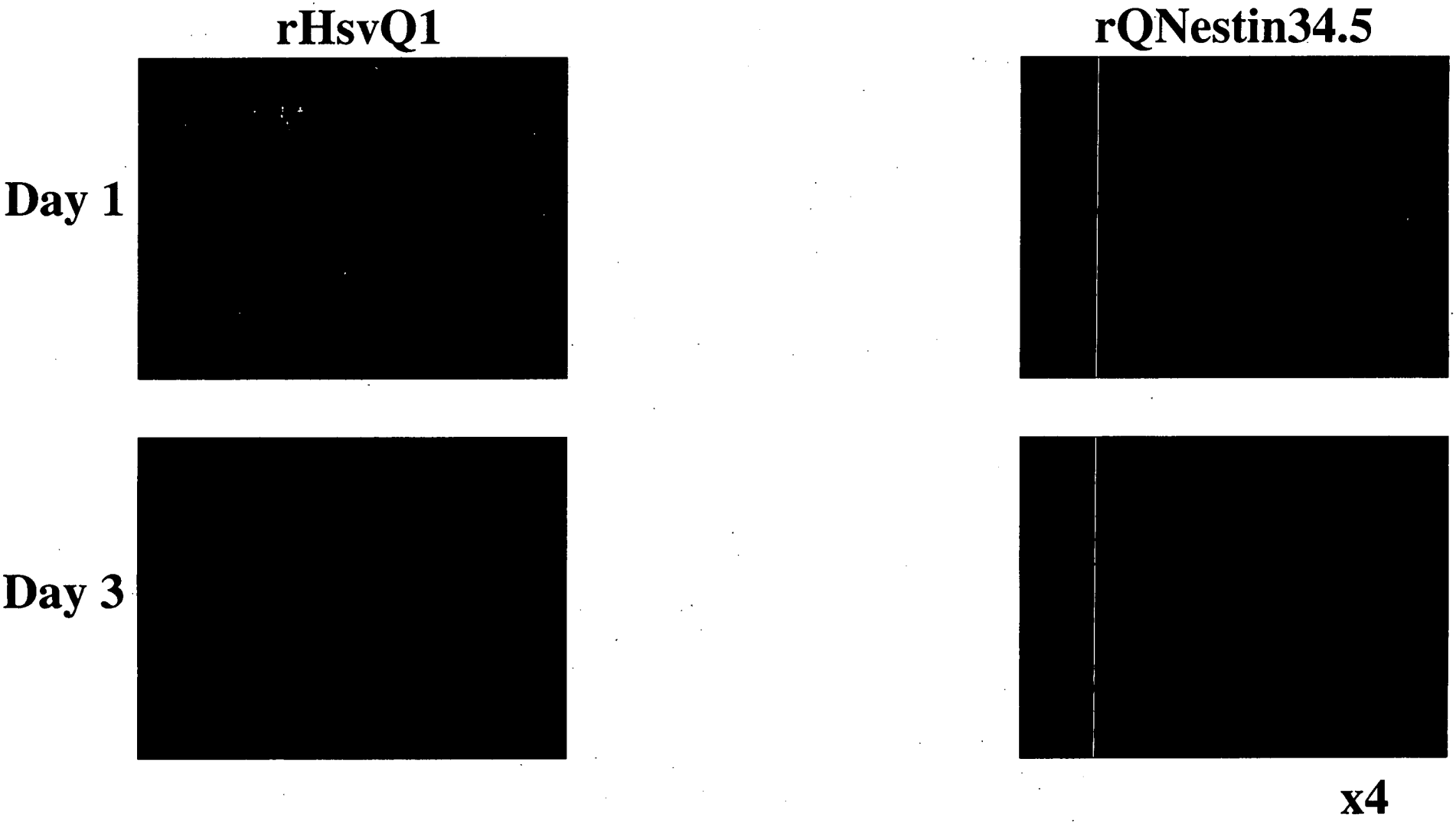


Fig.3C

T98G (low nestin) infected with $1x10^4$ pfus



Gli36ΔEGFR (low nestin) infected with $1x10^4$ pfus

Fig3D

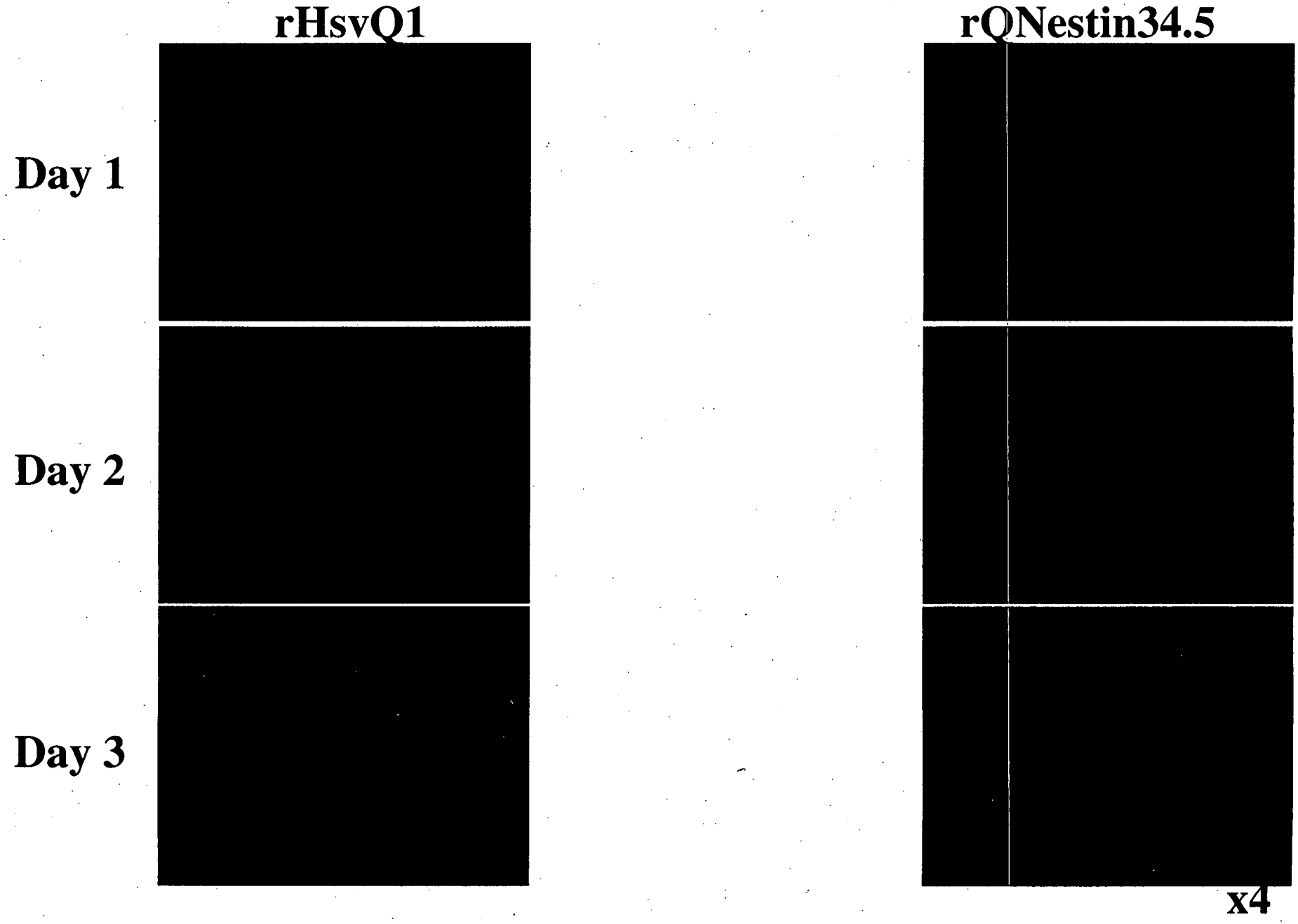
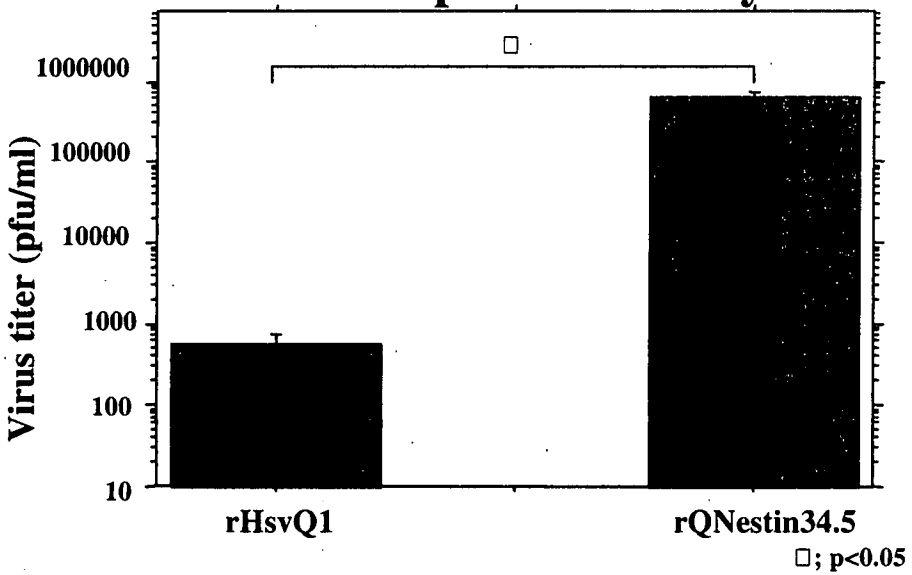


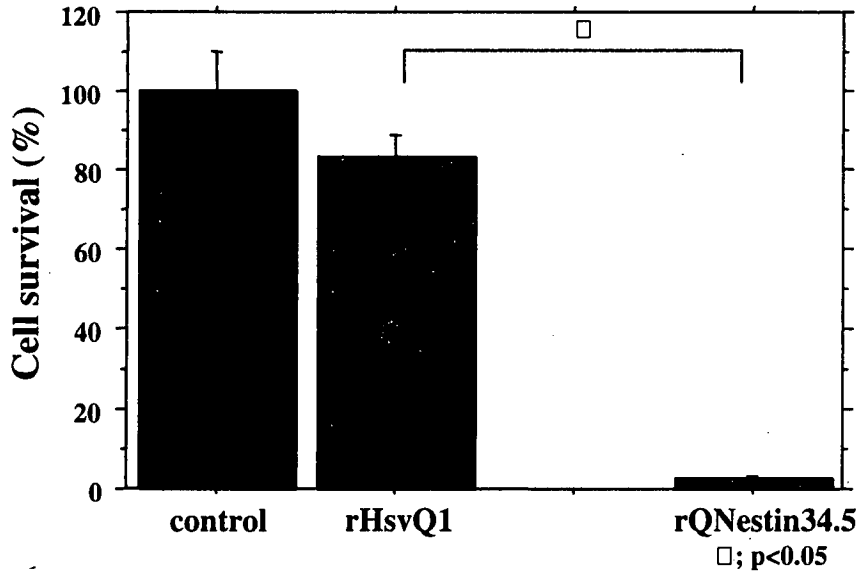
Fig 4A

U251 (high nestin)

Virus replication assay

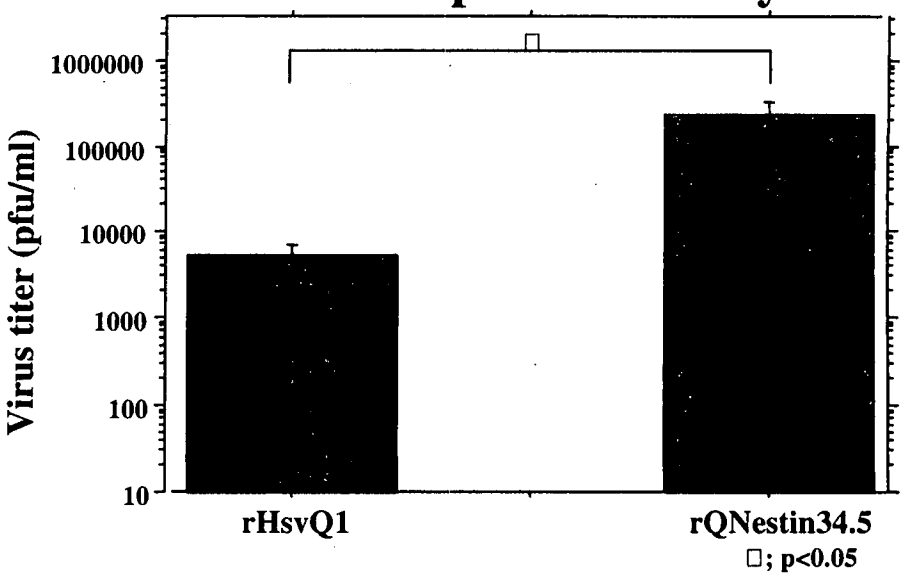


Cytotoxicity assay



U87ΔEGFR (high nestin)

Virus replication assay



Cytotoxicity assay

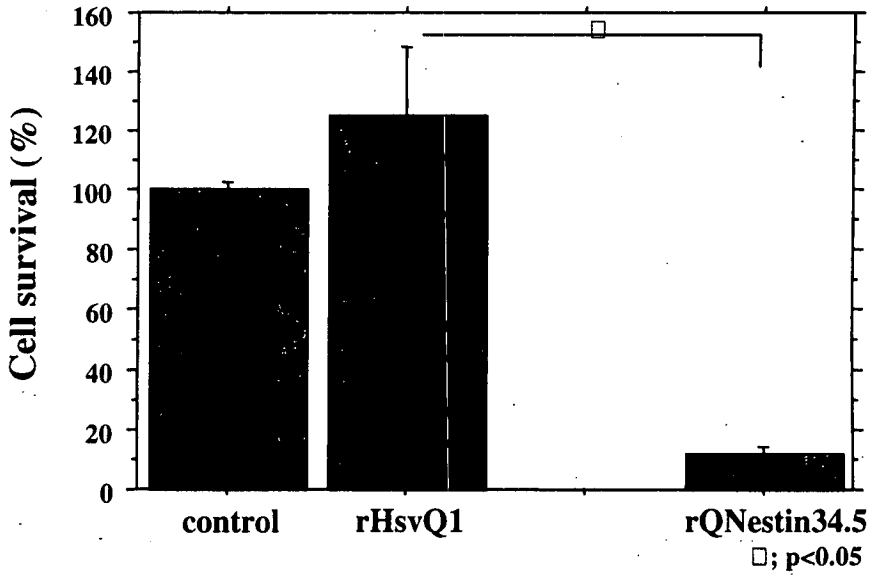


Fig. 4B

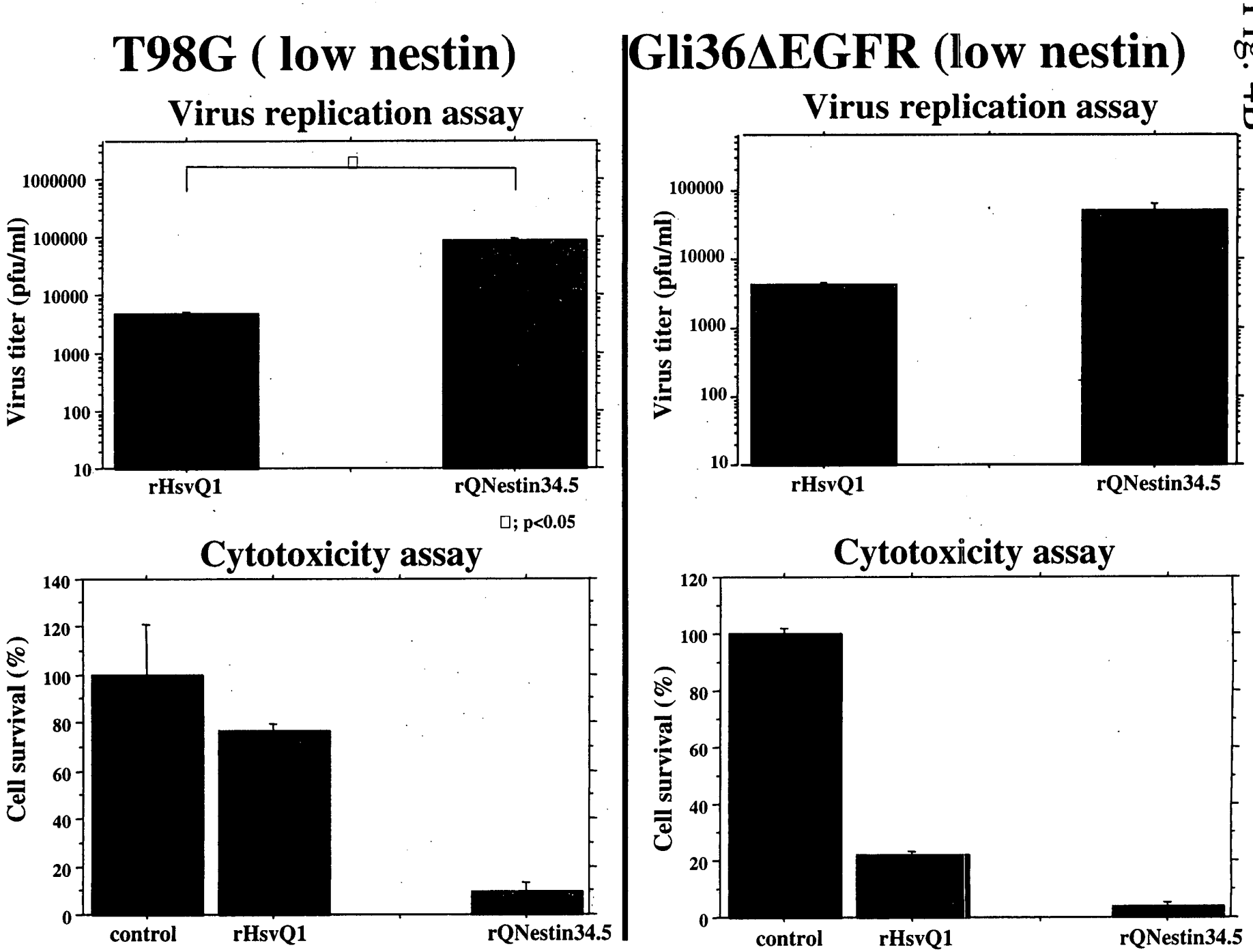
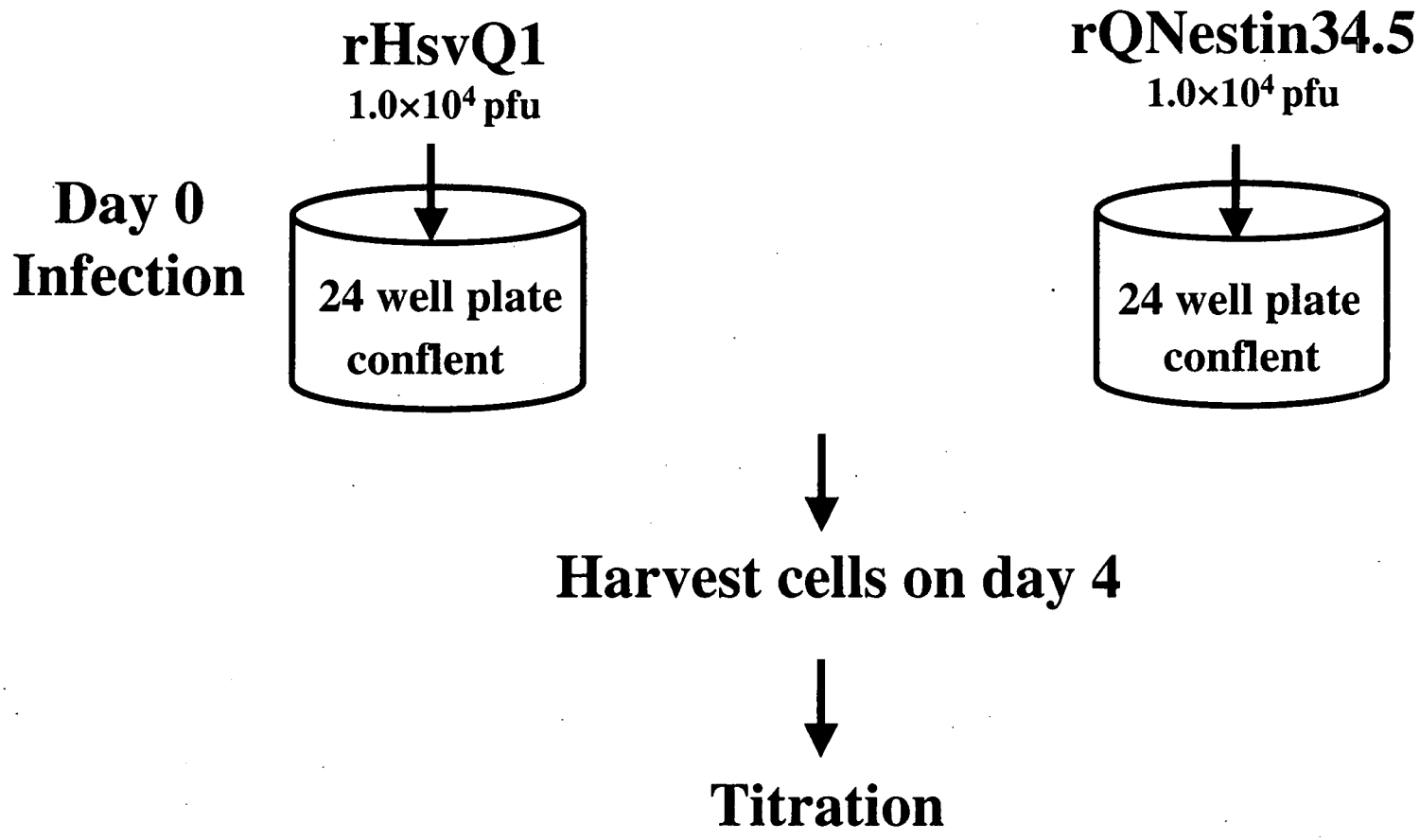

T98G (low nestin)
Virus replication assay
Virus titer (pfu/ml)
1000000
100000
10000
1000
100
10
rHsvQ1
rQNestin34.5
□; p<0.05
Cytotoxicity assay
Cell survival (%)
140
120
100
80
60
40
20
0
control
rHsvQ1
rQNestin34.5
Gli36ΔEGFR (low nestin)
Virus replication assay
Virus titer (pfu/ml)
100000
10000
1000
100
10
rHsvQ1
rQNestin34.5
Cytotoxicity assay
Cell survival (%)
120
100
80
60
40
20
0
control
rHsvQ1
rQNestin34.5

Fig. 5

Astrocytes

Virus replication assay



Astrocyte

Fig. 6

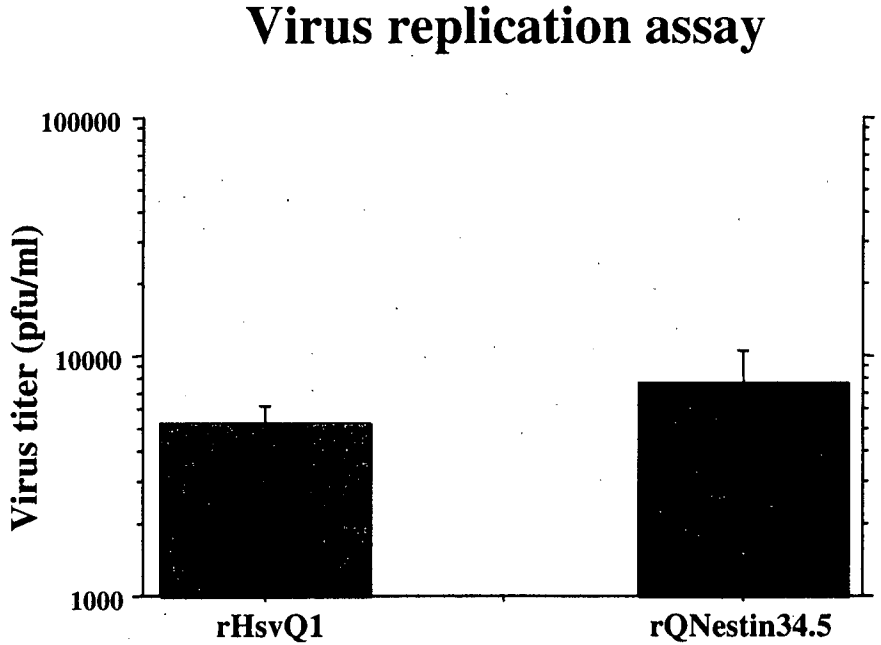
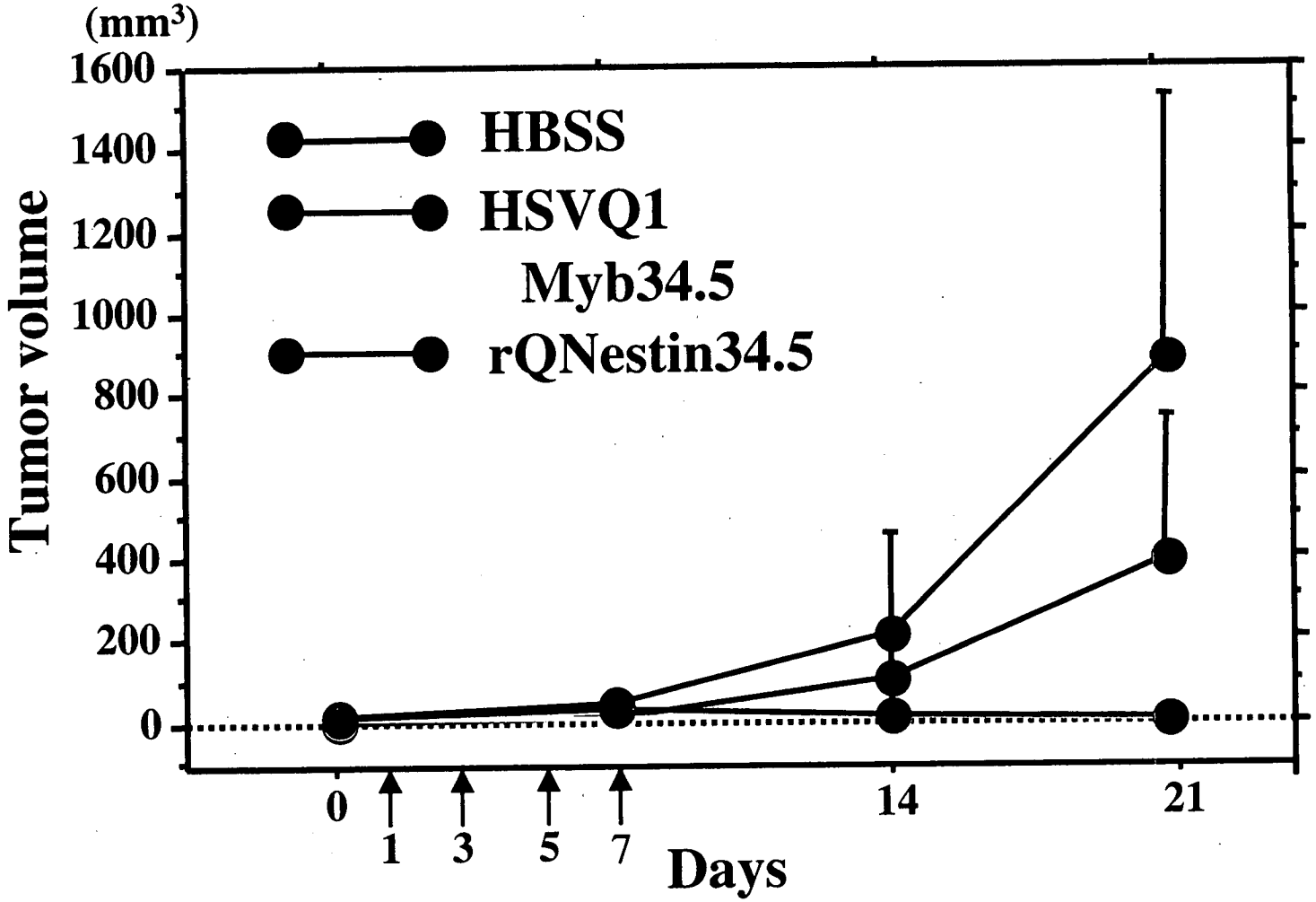


Fig. 7

In vivo growth inhibition
(U87ΔEGFR subcutaneus tumor model)



Oncolytic HSV

Fig. 8

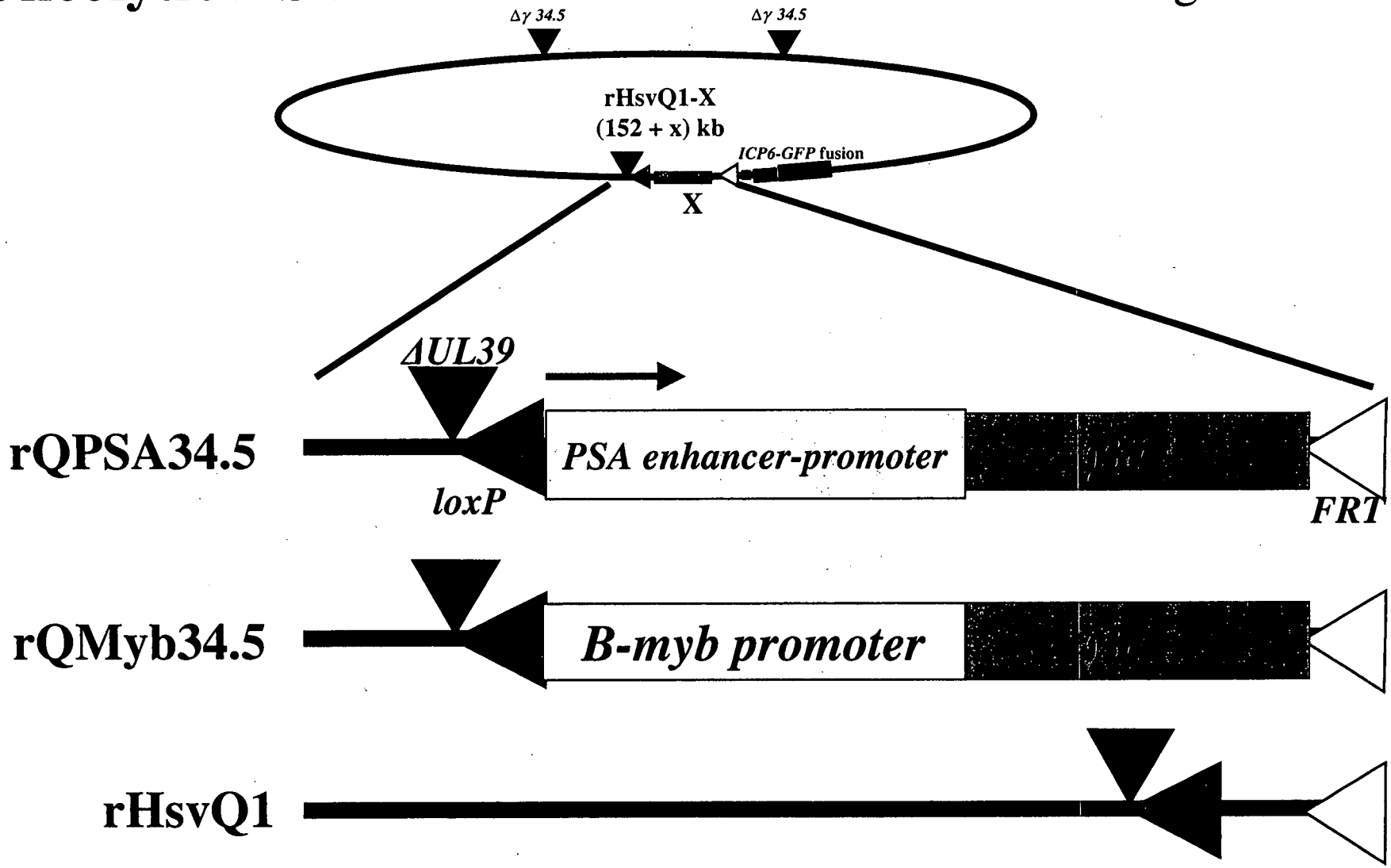


Fig. 9

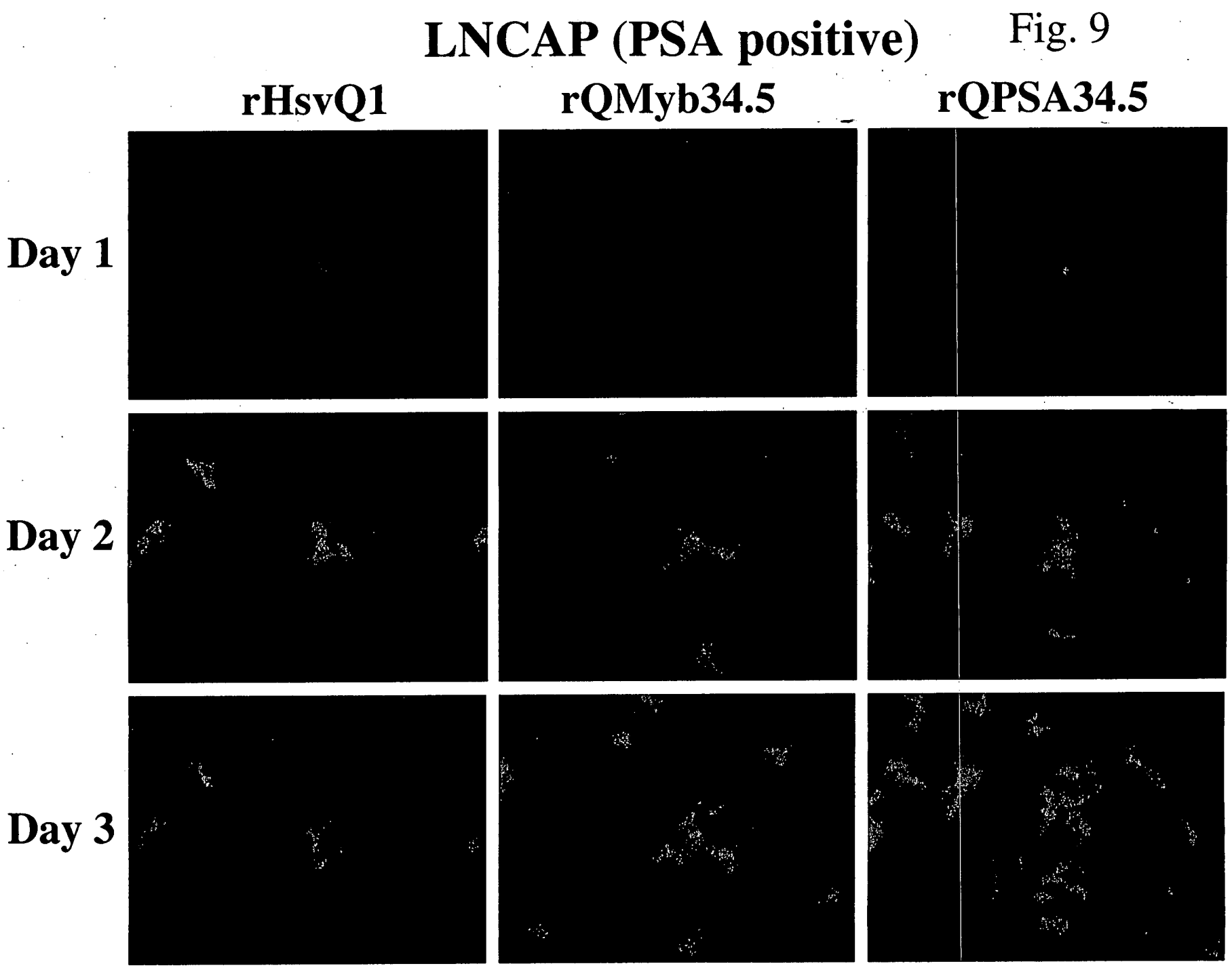

LNCAP (PSA positive)
rHsvQ1
rQMyb34.5
rQPSA34.5
Day 1
Day 2
Day 3

Fig. 10

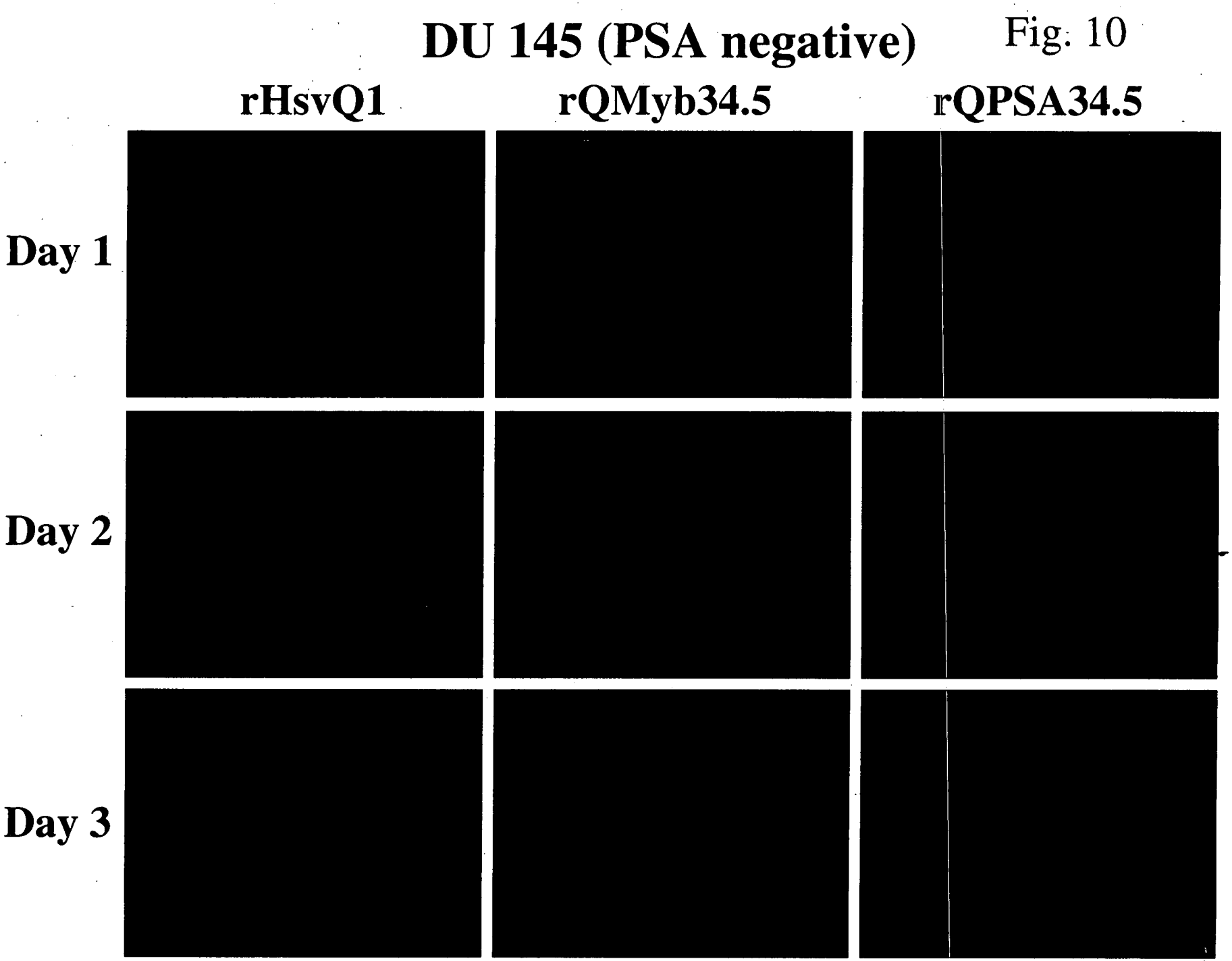
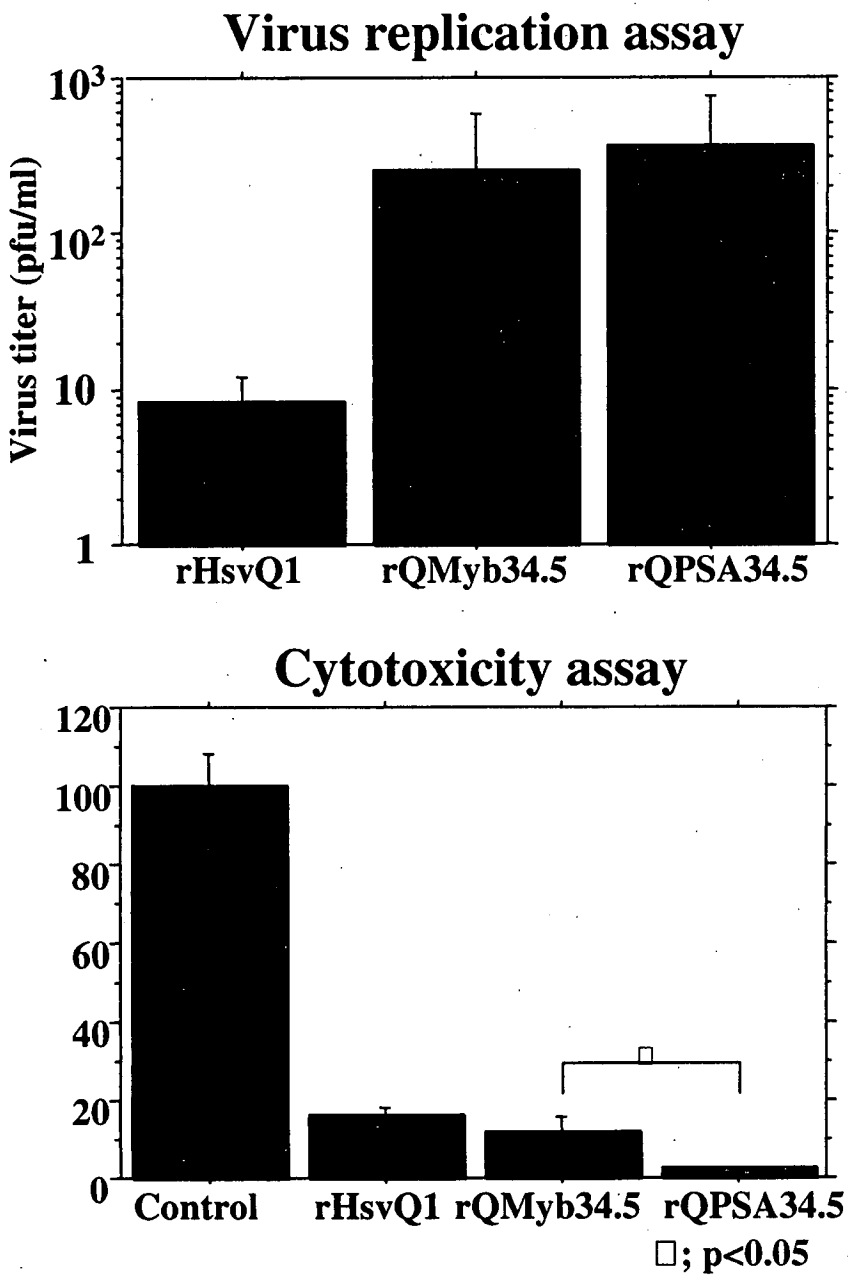

DU 145 (PSA negative)
rHsvQ1
rQMyb34.5
rQPSA34.5
Day 1
Day 2
Day 3

Fig. 11

LNCAP (PSA positive)



DU 145 (PSA negative)

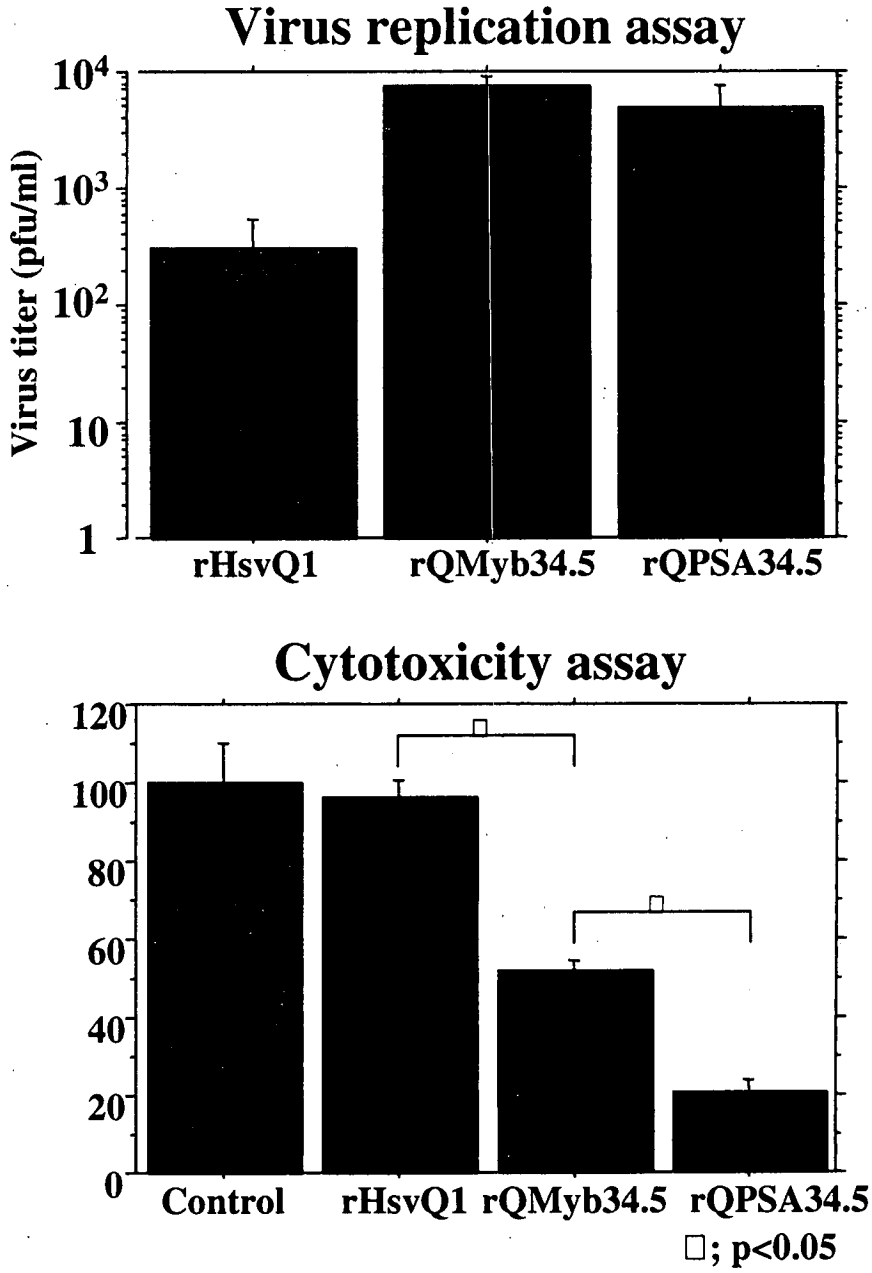


Exhibit-3

Exhibit 3

[CANCER RESEARCH 52, 5334-5341, October 1, 1992]

Expression of the Class VI Intermediate Filament Nestin in Human Central Nervous System Tumors[1]

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ABSTRACT

Tumor cells of a particular tissue may show a pattern of gene expression characteristic of the precursor cells of this tissue. To test this proposition for tumors of the central nervous system (CNS) we have used immunohistochemistry to analyze the expression of nestin in primary human CNS tumors and corresponding nonneoplastic brain tissue. Nestin defines a recently discovered sixth class of intermediate filament proteins and in the rat is expressed predominantly in CNS stem cells. In the adult nonneoplastic human brain we have detected only nestin expression in occasional endothelial cells. In contrast, a variety of primary CNS tumors contained substantially elevated nestin levels. The nestin-positive cells in the tumor tissue were tumor cells and/or endothelial cells. Glioblastomas expressed higher nestin levels than less malignant gliomas. This may indicate a correlation between nestin expression and malignancy within the glioma tumor group. In the primitive neuroectodermal class of tumors we observed both nestin-expressing and nonexpressing tumors, suggesting that nestin expression could be used to further characterize this complex and heterogeneous tumor group. Nine metastatic carcinomas were studied, and none showed nestin immunoreactivity in tumor cells. In conclusion, our data support the notion that primary CNS tumors share gene expression patterns with primitive, undifferentiated CNS cells and that nestin, like other intermediate filaments, may be useful in tumor diagnosis.

INTRODUCTION

There have been many proposals for the cellular origin of CNS[3] tumors. Bailey and Cushing (1) suggested that they were derived from an undifferentiated blast cell. A specific candidate for the tumor progenitor cells has been identified in the case of medulloblastomas, nests of undifferentiated cells, which normally give rise to the granular neurons of the cerebellum. Such nests have been demonstrated in otherwise normal, differentiated brain tissue (2). It might be expected that CNS tumor cells would show a gene expression pattern similar to that of the cells of the developing CNS from which they arise. Thus, the identification of RNA transcripts and proteins from such specifically expressed genes may be valuable in CNS tumor diagnosis, since they may be produced in CNS tumors only and not in corresponding adult nonneoplastic tissue.

Nestin is a gene which is potentially interesting in this respect. It encodes a newly discovered, sixth class of intermediate filament protein (3) and was originally detected by the monoclonal antibody Rat.401. By immunohistochemistry (4) and double-label fluorescence-activated cell sorting (5) nestin was shown to be expressed in rat CNS stem cells. Molecular characterization of the gene revealed a structural similarity (16–29% amino acid identity) to all previously characterized intermediate filament proteins (3). The similarity resides in an approximately 300-amino acid α-helical region required for filament formation (6, 7). This relatively low degree of amino acid identity and a variant mRNA splicing pattern justify the placement of nestin in a novel, sixth class of intermediate filaments (3, 8). The recent cloning of the human nestin gene reveals that it is distinctly similar to the rat gene, most notably in the α-helical region (9).

During CNS development the intermediate filament component of the cytoskeleton undergoes substantial remodeling. First, nestin is expressed in the CNS stem cells (3), and after its down-regulation, GFAP and neurofilaments are expressed in differentiated astrocytes and neurons, respectively (for a review, see Refs. 8 and 10). In addition, peripherin is expressed in subsets of neurons primarily in the peripheral nervous system (11), and internexin is transiently expressed in developing CNS (12).

Because of the strict temporal and spatial control of intermediate filament expression during development, they have been widely used to identify different cell types, both during normal development and in various tumors. Thus, identification of GFAP and neurofilaments have been used in the diagnosis of CNS tumors, and desmin and the cytokeratins in the diagnosis of rhabdomyosarcomas and carcinomas, respectively (see Ref. 13 for a review).

Based on the frequent use of intermediate filaments in tumor pathology, the evolutionary conservation of nestin between rat and humans (9), and the recent observation that neuroepithelium in developing human CNS transiently expresses nestin (14), we decided to analyze nestin expression in human CNS tumors and in adult nonneoplastic tissue.

MATERIALS AND METHODS

Antibodies. The two anti-nestin antisera 129 and 130 were produced in two separate rabbits immunized with a bacterially produced fusion protein containing the 4000 carboxy-terminal base pairs of the rat nestin gene (15, 16).[4] Monoclonal antibodies against GFAP (TpGFAP3) and the phosphorylated form of the M_r 200,000 (NFH) neurofilament (TpNFPIA3) have been described previously (17, 18).

Western Blot Analysis. Frozen tumor tissue was homogenized directly into sample buffer (19), and total protein was extracted. After shearing of high-molecular-weight DNA, separation of cellular debris by centrifugation, and boiling, approximately 2 μg of total protein were loaded in each lane of a 6% sodium dodecyl sulfate-polyacrylamide gel (19). The electrophoretically separated proteins were transferred to Immobilon P membranes (Millipore) using a BioRad transfer apparatus for 2 h at 90 V. After blocking in 3% bovine serum albumin for 1 h the filter strips were incubated overnight with the two polyclonal anti-nestin antisera or Coomassie blue stained to visualize the protein pattern. Antiserum 129 was used at a dilution of 1:600 and antiserum 130 at 1:2000 in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10

Received 4/29/92; accepted 7/27/92.

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[1] This work was made possible by grants from the Swedish Cancer Society, the Swedish Medical Research Council, Margaret och Axel Axön Johnsons Stiftelse, Knut and Alice Wallenbergs Stiftelse, Magn. Bergvalls Stiftelse, Stiftelsen Lars Hiertas Minne, Karolinska institutets fonder (J. D. and U. L.), and the Stockholm Cancer Society.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: CNS, central nervous system; PNE, primitive neuroectodermal; GFAP, glial fibrillary acidic protein; TBS, Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.6).

[4] M. Marvin and R. D. G. McKay, unpublished observations.

mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.2). The immunoreactivity was detected by a biotinylated anti-rabbit antibody and avidin-conjugated peroxidase as outlined below.

Tumor Classification and Preparation of Tissue Sections. The CNS tumors were classified according to the WHO (20), using the criteria of Burger *et al.* (21) to differentiate between glioblastomas and anaplastic astrocytomas. After surgery, biopsies were fixed in 10% formalin for approximately 24 h and then embedded in paraffin. Some tumors were fixed according to the B5 method (22). Sections (4 μm) were cut and mounted on microscope slides. The tissue sections were dried at 37°C for 4 h and then stored at −20°C until further use.

Immunohistochemical Staining Techniques. The mounted tissue sections were deparaffinated in xylene and then rehydrated through rinses in 99% and 70% ethanol. At this point, the slides were submerged into 70% ethanol with 5% iodine for 10 min, since some tumors had been fixed with B5 (22). After another rinse in 70% ethanol the slides were rinsed in water, and endogenous peroxidase was blocked by an incubation in 0.7% hydrogen peroxide in methanol, followed by a rinse in TBS and blocking for 30 min in 3% bovine serum albumin in phosphate-buffered saline. The slides were then incubated with first antibody for at least 12 h at room temperature. First antibodies were used at the following dilutions in phosphate-buffered saline: 129, 1:600; 130, 1:2000; TpGFAP3, 1:100; and TpNFPIA3, 1:50. After three rinses in TBS the second antibody was added and incubated for 60 min (biotinylated swine anti-rabbit Ig, Dakopatts E 353 at 1:500 for 129 and 130; biotinylated rabbit anti-mouse Ig Dakopatts E 354 at 1:250 for TpGFAP3 and TpNFPIA3). Following three rinses in TBS a premixed complex of horseradish peroxidase-conjugated avidin and biotin was added in 50 mM Tris pH 7.6 (Dakopatts K 355) and incubated for 30 min. After three rinses in TBS, diaminobenzedine at 0.6 mg/ml in TBS was added for 10 min, and the reaction was terminated by a quick rinse in TBS, followed by rinses in water and 70% and 99% ethanol. Some sections were counterstained with haematoxylin. Finally, coverslips were mounted with Mountex (Histolab AB). The sections were inspected under a Leitz light microscope and photographed at ×100 magnification.

Double Immunofluorescence Experiments. Tumor sections were treated as above for hydration and blocking. Sections were then incubated overnight with anti-nestin antiserum 130 at 1:500, rinsed three times in TBS as above, and incubated with fluorescein-conjugated sheep anti-rabbit IgG antiserum (Boehringer 1238 833, diluted 1:80) for 60 min. After three rinses in TBS the sections were incubated overnight with the monoclonal TpGFAP3 antibody (1:250). Following three rinses in TBS the sections were finally incubated with rhodamin-conjugated sheep anti-mouse IgG antiserum (Boehringer 1214 608, diluted 1:80) for 60 min. The sections were analyzed and photographed for fluorescein and rhodamin fluorescence under a Nikon microphot microscope at ×100 and ×200 magnification. Control experiments were performed in which the anti-nestin and the anti-GFAP antibodies were omitted in separate experiments. No cross-binding between the second antibodies could be detected.

Northern Blots. Polyadenylated RNA (1.25 μg) from human adult brain (a 21-year-old, disease-free male) was denatured and electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde in 1× 4-morpholinepropanesulfonic acid buffer for 4 h (23). The RNA was then transferred to Nylon membrane (Hybond-N; Amersham). The filter was prehybridized and hybridized (5× standard saline citrate, 5× Denhardt's, 250 mg/ml salmon sperm DNA, 50% formamide, 50 mM sodium phosphate [pH 6.5], 0.5% sodium dodecyl sulfate at 42°C for 16 h) with 2×10^6 cpm/ml of a mixture of two different ^{32}P-labeled probes (24) of the human nestin gene (9): the first derived from 900 base pairs of the first exon (starting from 100 nucleotides upstream of the transcription start) and the second from 3240 base pairs from the carboxy-terminal region (corresponding to amino acids 375 to 1453). After hybridization the filter was washed (final wash, twice for 30 min each at 65°C in 0.2 × standard saline citrate, 0.2% sodium dodecyl sulfate) and exposed to X-ray film at −70°C for 14 days with intensifying screens. The size of the hybridizing mRNA was determined by running RNA molecules of known size (BRL) in parallel.

RESULTS

Characterization of the Two Anti-Nestin Antisera

Sections from a variety of CNS tumors and nonneoplastic adult brain tissue were analyzed by immunohistochemistry with antibodies to the intermediate filaments GFAP, neurofilament, and nestin. The two antibodies against nestin, 129 and 130, are specific for nestin in rat (16) and recognize a molecule of similar size in human material (14, 16). To confirm that the antibodies truly recognized the equivalent human protein in our tumor material we performed Western blot analysis of an immunohistochemically nestin-positive tumor. The antisera identified a protein in the expected molecular weight range (Fig. 1*a*). Fig. 1*b* shows the intermediate filament-like staining pattern, observed for all nestin-positive cells in the various tumors. Antisera 129 and 130 gave similar staining patterns in the tumors, while the incidence of positive cells could vary somewhat. This may be a consequence of differences between different sections or slight differences between the two antisera (Table 1). Preimmune serum from the same rabbits, used as a control under identical conditions, did not give any reaction (data not shown).

Staining of Nonneoplastic Adult Human Brain Tissue

Sections from brain tissue removed in the treatment of epilepsy from six patients were analyzed for nestin, GFAP, and neurofilament expression. None of the cases were tumor related. The expected neurofilament and GFAP staining was easily identified in neuronal fibers and astrocytes, respectively (Fig. 2, *a* and *b*). High levels of GFAP expression in reactive astrocytes were seen in some cases and were probably related to the epileptic focus.

In contrast, only a very small number of cells reacted with the anti-nestin antibodies, and these were endothelial cells (Table 1). In the positive cells the intensity of the immunoreactivity was very low (Fig. 2*c*). Nestin-positive cells were identified in only three of the specimens, one each from the hippocampus, frontal lobe, and lateral neocortex. In the other three specimens, taken from temporal lobe, temporal cortex, and lateral neocortex, no nestin immunoreactivity was detected. Even in regions that contained nestin-positive endothelial cells, the vast majority of endothelial cells were negative for nestin expression. Neither preimmune serum nor the monoclonal antibodies

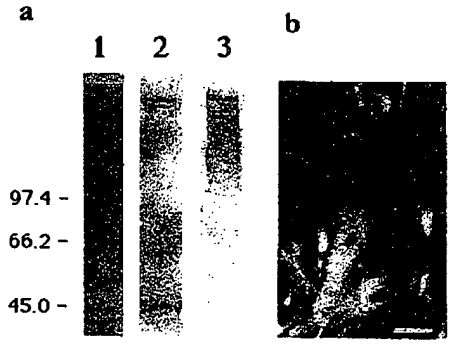

Fig. 1. Identification of nestin in Western blots and in tumor sections. In *a*, 2 μg of total protein from a glioblastoma tumor (Table 1, tumor 12) were Western blotted with the anti-nestin antisera 129 (*Lane 2*) and 130 (*Lane 3*). *Lane 1*, a strip from the same filter stained with Coomassie blue. Indicated molecular weights are in kilodaltons. *b*, immunofluorescence microscopy of a glioblastoma tumor section (Table 1, tumor 7) with anti-nestin antiserum 130. Cytoplasmic staining with an intermediate filament-like pattern can be observed. *Bar*, 10 μm.

Table 1 *Immunohistochemical staining of control brain and tumors with antibodies to nestin and GFAP*

Case		Age	Nestin 129	Nestin 129	Nestin 130	Nestin 130	GFAP
Nonneoplastic brain							
	Region		Neuro ect.	Endo	Neuro ect.	Endo	Astro-cytes
	l.n.[a]	30	0[b]	*	0	*	*
	l.n.	30	0	0	0	0	*
	r.t.c.	47	0	0	0	0	*
	r.t.l.	10	0	0	0	0	*
	f.l.	11	0	*	0	*	*
	h.	30	0	*	0	*	*
Primary tumors							
	Type		Tum.	Endo.	Tum.	Endo.	Tum.
1	GB[c]	67	2	0	1	0	1
2	GB	65	2	1	2	0	2
3	GB	56	1	1	2	1	2
4	GB	45	1	0	1	1	2
5	GB	32	3	0	3	0	2
6	GB	68	3	0	3	0	1
7	GB	61	2	2	2	2	1
8	GB	74	2	1	1	1	1
9	GB	59	2	0	2	0	1
10	GB	60	2	1	2	2	1
11	GB	50	2	2	2	2	1
12	GB	44	2	2	1	2	1
13	GB	56	1	0	2	0	1
14	GB	41	1	1	1	1	1
15	GB	19	1	0	2	0	0
16	AA	37	0	2	0	2	2
17	AA	29	0	1	0	1	2
18	AA	66	0	0	0	0	1
19	AA	44	1	0	1	0	2
20	AA	20	3	0	3	0	1
21	AMG	17	0	0	1	0	1
22	A	54	0	2	0	2	2
23	A	11	0	0	1	0	1
24	A	48	0	1	0	1	1
25	A	44	0	2	0	2	0
26	A	27	0	1	0	1	2
27	A	43	0	0	0	1	1
28	A	36	1	1	2	1	1
29	AO	17	0	2	0	2	1
30	AO	35	1	1	1	1	2
31	AO	24	1	1	1	2	2
32	AO	54	0	1	0	1	1
33	AO	67	1	1	1	1	1
34	O	68	0	0	0	0	0
35	O	14	1	1	1	1	0
36	O	32	0	1	0	1	1
37	O	29	1	1	0	1	0
38	O	47	1	1	1	1	2
39	AE	33	1	1	1	1	2
40	E	10	0	2	0	1	1
41	E	17	1	0	1	0	1
42	E	9	1	0	1	0	0
43	E	23	0	0	0	0	0
44	E	22	0	0	0	1	0
45	E	1	1	1	1	1	1
46	PA	14	0	2	0	1	1
47	PA	5	0	0	0	0	0
Primary tumors cont'd							
48	PA	10	0	0	0	0	0
49	PA	3	0	0	0	0	1
50	PA	12	0	1	0	1	1
51	PA	3	0	0	0	0	1
52	PNE	10	0	2	0	2	2
53	PNE	33	2	2	2	2	2
54	PNE	8	0	0	0	0	0
55	PNE	2	0	2	1	2	1
56	PNE	11	0	1	0	1	0
57	PNE	13	0	1	0	1	0
58	PNE	6	1	1	1	1	1
59	PNE	10	0	0	0	0	0
60	PNE	1	1	1	1	2	0
61	PNE	4	0	0	0	1	0
62	PNE	6	0	0	0	0	0
63	PNE	11	0	0	0	0	0
64	PNE	4	0	0	0	0	0
65	PNE	4	0	0	0	1	0
66	PNE	2	1	0	1	0	0
67	PP	19	0	0	0	0	0
Metastases							
68	LDC	71	0	0	0	0	0
69	LDC	65	0	2	0	2	0
70	LDC	56	0	0	0	0	0
71	LDC	64	0	1	0	1	0
72	LDC	57	0	1	0	1	0
73	LDC	67	0	0	0	1	0
74	LDC	69	0	0	0	1	0
75	LDC	69	0	2	0	2	0
76	LDC	61	0	0	0	0	0
77	Rha	10	1	0	1	0	0
78	Nb	2	0	0	0	0	0

[a] Abbreviations for adult control brain: l.n., lateral neocortex; f.r.l., frontal lobe; r.t.l., right temporal lobe; r.t.c., right temporal cortex; h. (hippocampus). Nestin staining in endothelial (endo) and tumor cells (tumor) is denoted separately. *, presence of occasional nestin-expressing cells in adult tissue (nest.) or astrocytes expressing GFAP. Data are from immunohistochemical analysis (avidin-biotin-peroxidase complex) of 67 tumors of CNS origin, 10 tumors that have metastasized to the brain, and one retroperitoneal neuroblastoma.

[b] Staining patterns: 0, no staining; 1, staining in less than 5% of cells in the tumor; 2, staining in 5–50%, 3, staining in more than 50%. For nestin the staining results from the two antibodies (129 and 130) in tumor cells (tumor) and endothelial cells (endo) are denoted separately. GFAP staining (GFAP tum.) is only denoted when it occurs in tumor cells, not in astrocytes.

[c] Abbreviations for tumors: GB, glioblastoma, malignancy grade IV; AA, anaplastic astrocytoma, grade III; AMG, anaplastic mixed glioma, grade III; A, astrocytoma, grade II; AO, anaplastic oligodendroglioma, grade III; O, oligodendroglioma, grade I–II; AE, anaplastic ependymoma, grade III; E, ependymoma, grade II; PA, pilocytic astrocytoma, grade I; PNE, primitive neuroectodermal tumors, grade IV; PP, plexus papilloma; LDC, low differentiation carcinoma; Rha, rhabdomyosarcoma; Nb, retriperitoneal neuroblastoma.

to GFAP or neurofilament protein resulted in staining of endothelial cells. Morphological inspection of adjacent hematoxylin-eosin counterstained sections revealed that none of the cases exhibited any signs of neoplastic change.

Nestin expression in adult brain was analyzed also at the RNA level. When a Northern blot filter of polyadenylated RNA from adult human brain was hybridized with a probe from the cloned human nestin gene, we observed a weakly hybridizing band, corresponding to a 6-kilobase mRNA species (Fig. 3).

Nestin Staining in Tumors

Sectioned material from the surgical specimens of 78 formalin-fixed tumors was analyzed with the antibodies to nestin, GFAP, and neurofilament protein. The specimens consisted of 67 primary tumors, 10 metastatic tumors to the CNS (nine low differentiated carcinomas of known origin and a single metastatic rhabdomyosarcoma), and one primary retroperitoneal neuroblastoma. In total, nestin expression was detected in the tissue of 62 of the tumors (Table 1). The nestin immunoreactivity was detected only in two cell types: endothelial cells and tumor cells.

Endothelial Cells. Forty-seven tumors (60%), of both primary and metastatic origin, contained nestin-positive endothelial cells (Fig. 4*a*). Nestin immunoreactivity was most intense in proliferating endothelium, particularly in the malignant gliomas, but was detected in all types of primary CNS tumors analyzed. The single neuroblastoma and the single plexus papilloma showed no immunoreactivity for nestin (Table 1). In some tumors nestin expression was confined to endothelial cells, whereas other tumors contained both nestin-positive endothelial and tumor cells (see below).

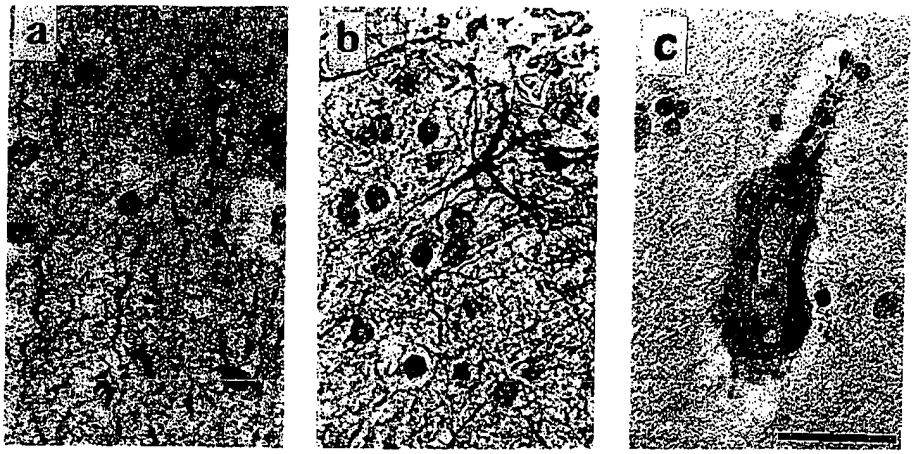

Fig. 2. Immunostaining of adult, nonneoplastic human brain. A section from an adult epileptic neocortex was immunostained (avidin-biotin-peroxidase complex) with antsiera against M_r 200,000 neurofilament protein (*a*), GFAP (*b*), and nestin 130 (*c*). Immunoreactivity is noted in nerve fibers (neurofilament), reactive astrocytes (GFAP), and endothelial cells (nestin). *Bar*, 100 μm.

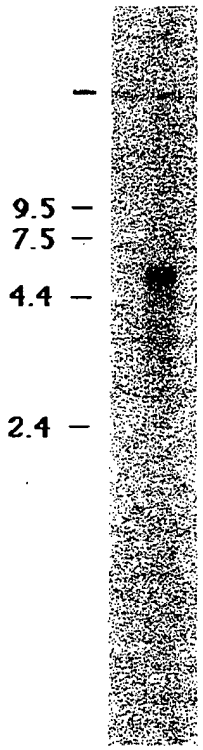

Fig. 3. Expression of the human nestin gene in adult brain. Northern blot analysis of 1.25 μg polyadenylated RNA from adult human brain. The blot was probed with a portion of the cloned human nestin gene and exposed with an intensifying screen for 14 days.

Tumor Cells. Nestin-positive tumor cells were detected in 36 (46%) of the 78 tumors. Staining was observed only in tumor cells of primary CNS tumors and not in carcinoma metastases. In 14 of the 36 tumors nestin immunoreactivity was confined to tumor cells (Fig. 4*b*), and in the remaining 22 tumors, positive tumor cells were found together with positive endothelial cells (Fig. 5, *a* and *c*). Tumor cells strongly expressing nestin were often found in close proximity to blood vessels (Fig. 5*c*). Nestin-positive tumor cells typically had very irregular shapes, often with cellular extensions up to several cell diameters in length (Figs. 4*b* and 5*c*). The positive tumor cells were often confined to certain regions of a tumor section, with relatively sharp demarcations of surrounding negative tumor tissue (Fig. 4*c*). In addition, nestin-positive multinucleated cells and cells undergoing cell division were frequently found (Fig. 4*b*).

Patterns of Nestin Expression in the Various Tumor Types

Gliomas. Tumors from nine types of gliomas were analyzed, and nestin immunoreactivity could be detected in all classes, although to varying degrees (Table 1). Glioblastomas (malignancy grade IV) expressed the highest incidence of nestin-positive cells and in general the highest levels of nestin staining. Of the 15 cases analyzed all expressed nestin in tumor cells and nine in endothelial cells, and in most cases the immunostaining was observed in a large number of cells in the tumor (Table 1). Five cases of anaplastic astrocytoma (malignancy grade III) were analyzed. Two expressed nestin only in tumor cells, and two others only in endothelial cells. In the single case of anaplastic mixed glioma (malignacy grade III) only a few positive tumor cells were found. Astrocytomas (malignancy grade II) contained nestin-positive tumor cells in two and endothelial cells in six of seven tumors. Anaplastic oligodendrogliomas (malignancy grade III) expressed nestin in tumor cells in three of five tumors, whereas in all five cases the endothelial cells were positive. In the five cases of oligodendrogliomas (malignancy grades I-II) we found nestin-immunoreactive tumor cells in three tumors and endothelial cells in four cases. The single case of anaplastic ependymoma (malignancy grade III) contained both nestin-immunoreactive tumor cells and endothelial cells. Three of the six cases of ependymomas (malignancy grade II) were nestin-positive in tumor cells and three in endothelial cells. No nestin-positive tumor cells were detected in the six cases of pilocytic astrocytomas (malignancy grade I), while two of these tumors contained nestin-positive endothelial cells.

PNE Tumors. Fifteen cases of PNE tumors were analyzed. Five of these showed nestin immunoreactivity in tumor cells and nine in endothelial cells. Four of the latter were also positive in tumor cells (Table 1). Analysis of GFAP immunoreactivity revealed that 4 of the 15 tumors showed GFAP-positive tumor cells. Eight tumors contained neurofilament-positive cells (data not shown).

Other Primary Tumors. No detectable levels of nestin were expressed in the single cases of neuroblastoma (retroperitoneal tumor) and plexus papilloma tumors analyzed.

Metastases. We analyzed ten cases of metastases to the brain: nine carcinomas of known origin and one rhabdomyosarcoma (Table 1). The rhabdomyosarcoma tumor tissue showed expression of nestin in tumor cells but not in endothelial cells.

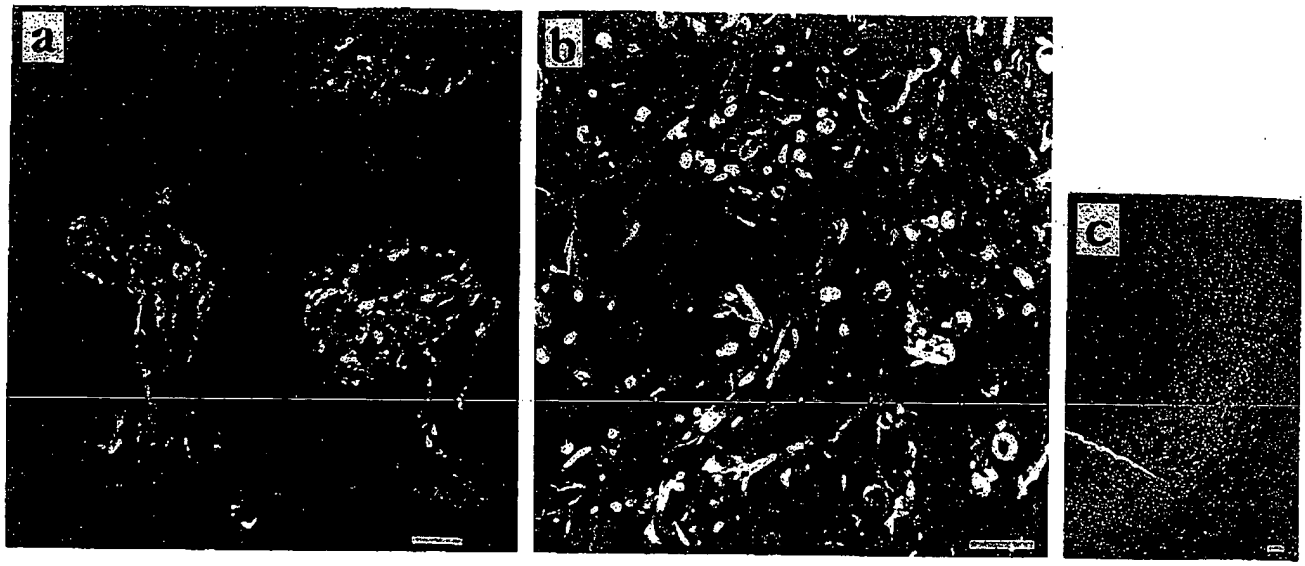

Fig. 4. Expression of nestin in endothelial and tumor cells. Immunofluorescence microscopy of tumor sections with anti-nestin antiserum 130. *a*, immunopositive endothelial cells in a metastasizing tumor (Table 1, tumor 75). Note that only endothelial cells stain with the anti-nestin antiserum. *b*, immunopositive tumor cells in a glioblastoma tumor (Table 1, tumor 9). Multinucleated tumor cells are positive. In *c*, a lower magnification of *b* is included to demonstrate the variation of immunopositivity for nestin in the tumor. *Bar*, 100 μm (*a* and *b*), 1000 μm (*c*).

Of the other nine metastases none contained nestin-positive tumor cells, whereas six contained immunoreactive endothelial cells.

Nestin Staining in Tissue Adjacent to CNS Tumors

We analyzed the expression of nestin, GFAP, and neurofilament in sections taken from regions in close proximity to the tumor tissue of four astrocytomas. The overall morphology was nontumoral, as determined from adjacent hematoxylin-eosin sections (data not shown). This was evident also from the patterns of GFAP and neurofilament immunoreactivity: reactive astrocytes with increased GFAP levels and ordered neurofilament-positive nerve fibers were seen (Fig. 6, *a* and *b*). In these sections we found low levels of nestin staining only in a proportion of the endothelial cells (Fig. 6*c*). This was, however, somewhat more intense than that found in control brain but less intense than in tumor tissue.

Double-Label Immunofluorescence with Nestin and GFAP

The peroxidase staining suggested that nestin and GFAP in certain tumors had very similar expression patterns. To investigate in more detail the possible coexpression of nestin and GFAP we analyzed their intracellular distribution by double immunofluorescence. In some tumors the distributions of nestin and GFAP were quite distinct, *e.g.*, where nestin was mainly expressed in endothelial cells and GFAP only in astrocytes in the same tumor (Fig. 5, *a* and *b*). This demonstrates the specificity of each antibody for its target intermediate filament protein. In certain tumors, however, many cells coexpressed nestin and GFAP, in particular tumor cells surrounding small blood vessels, whereas in adjacent endothelial cells nestin alone was expressed (Fig. 5, *c* and *d*). At high magnification it was apparent that the intracellular distribution in coexpressing cells is very similar (Fig. 5, *e* and *f*).

DISCUSSION

The rat nestin gene encodes a recently discovered intermediate filament protein expressed predominantly in CNS stem cells (3–5). Stimulated by the long-standing interest in the relationship between undifferentiated cells of the developing CNS and CNS tumor cells (1, 25) and the fact that other intermediate filaments have significantly contributed to the diagnosis of various tumors (13), we have analyzed nestin expression in human CNS tumors and control nonneoplastic brain tissue.

In the adult, nonneoplastic human brain nestin immunoreactivity was detected only in occasional endothelial cells in the frontal lobe, hippocampus, and lateral neocortex. A small number of cells expressing nestin at a low level is supported by our observation of very low nestin mRNA levels in RNA from adult brain. Our findings extend a recent analysis of nestin expression during human embryonic CNS development (14). Tohyama *et al.* (14) observed a transient nestin expression in human neuroepithelium but noted in addition an endothelial expression pattern that was maintained until at least 40 weeks after gestation. Taking into account our finding of sporadic adult expression in endothelial but not in the neuroectodermally derived CNS cells in the adult brain, the available data suggest that human brain nestin expression occurs (*a*) as a transient phase in embryonic neuroectodermal development and (*b*) probably throughout life in the endothelial component. Nestin expression in the endothelial cell may be related to proliferation, as discussed below.

Increased Nestin Expression in CNS Tumors. In contrast to the rare findings of nestin immunopositivity in endothelial cells of adult nonneoplastic brain, we detected considerably elevated nestin immunoreactivity in a variety of CNS tumors. Both endothelial cells and tumor cells expressed nestin. Nestin expression in endothelial and tumor cells is presumably independently regulated, since tumors containing positive cells of both, one, or neither cell type were found.

Our data indicate that the nestin expression in endothelial cells may be correlated with the degree of cellular proliferation. First, the most intensely nestin-immunoreactive cells were found in the proliferating endothelium of tumors, frequently of the glioblastomas. Second, in nonneoplastic brain tissue, where proliferation occurs at very low rates, only occasional endothelial cells expressed nestin, and then at low levels. Third, in

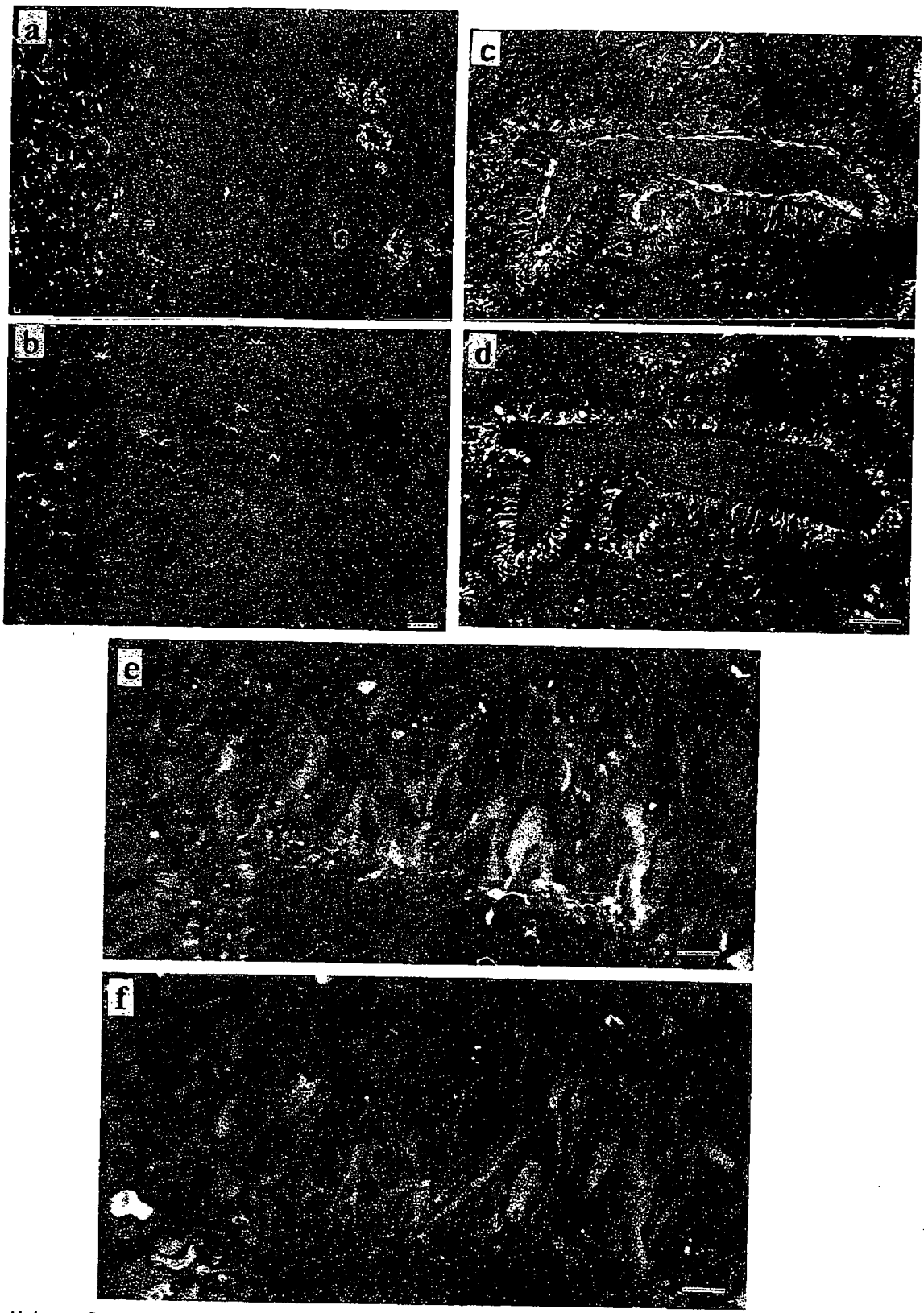

Fig. 5. Double-immunofluorescence studies of tumors with anti-GFAP and anti-nestin antisera. Sections from an anaplastic astrocytoma (*a* and *b*) (Table 1, tumor 16), a glioblastoma (*c* and *d*) (Table 1, tumor 11), and a PNE tumor (*e* and *f*) (Table 1, tumor 53) were double-stained with antisera to nestin 130 (*a*, *c*, and *e*) and GFAP (*b*, *d*, and *f*). The staining of proliferating endothelial cells for nestin (*a*) and probably reactive astrocytes for GFAP (*b*) can be observed. In *c* the endothelial cells are stained only by nestin, and the tumor cells by both nestin (*c*) and GFAP (*d*). The similarities of the intracellular nestin (*e*) and GFAP (*f*) staining patterns are shown at higher magnification. Occasional cells stain with only one of the antisera. *Bar*, 200 μm (*a* and *b*), 50 μm (*c* and *d*), and 20 μm (*e* and *f*).

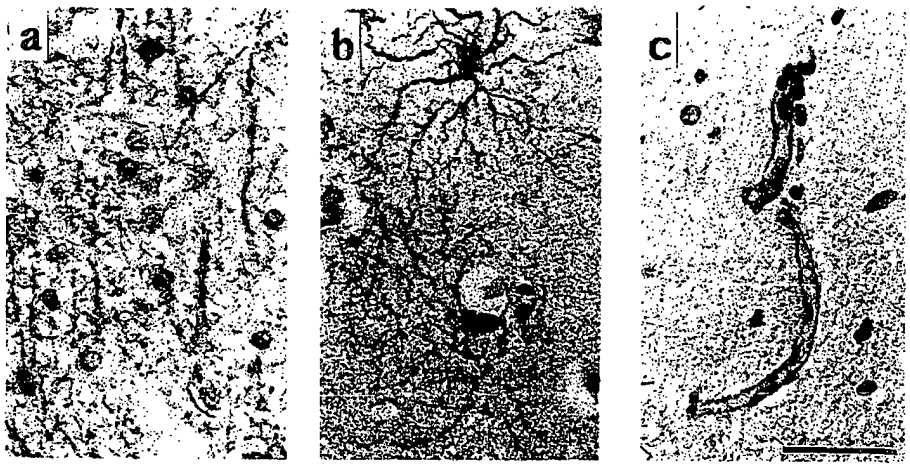

Fig. 6. Expression of nestin in tissue adjacent to a tumor. M_r 200,000 neurofilament protein (*a*), GFAP (*b*), and nestin 130 (*c*) immunostaining (avidin-biotin-peroxidase complex) of part of a section taken from the periphery of an astrocytoma (malignancy grade II). *a*, neurites; *b*, probable reactive astrocytes. Nestin staining is noted only in some endothelial cells (*c*). *Bar*, 100 μm.

reactive nonneoplastic tissue from the immediate vicinity of tumors, where increased endothelial proliferation may occur, endothelial cells also expressed nestin, the percentage of positive cells being intermediate between nonneoplastic control brain and the highly proliferating endothelium of tumors. In addition, in some carcinoma metastases nestin immunoreactivity was readily detected in endothelial cells. The endothelial cells are not themselves neoplastic, but their degree of proliferation may be subject to control by angiogenic factors produced by adjacent tumor cells. It has been shown that CNS cells grown in primary culture respond to the angiogenic factor basic fibroblast growth factor, in synergy with nerve growth factor, by maintaining high levels of nestin protein (26). However, the absence of nestin-positive cells in the endothelium of some of the very malignant tumors, where angiogenesis would be expected to occur, suggests that the correlation between nestin expression and endothelial proliferation is not absolute.

In contrast to the endothelial expression pattern, where nestin apparently can be directly or indirectly induced by adjacent tumor cells, the neuroectodermally derived cells seem to be more selective in their nestin expression. Our data suggest that glial cells do not express nestin except when transformed. No nestin expression could be detected in the reactive glioses surrounding the tumors or in relation to some of the epilepsy foci. This implies that exposure to growth and other factors secreted by the tumor cells does not induce nestin expression in adult glial cells, while expression in endothelial cells appears to be affected by such factors. Furthermore, nestin expression was detected in tumor cells of various types of primary CNS tumors, which are of neuroectodermal origin, but not in metastasizing carcinoma cells. Tumor cells of the rhabdomyosarcoma, a tumor of muscle origin, expressed nestin. This is to be expected, since it has been established that developing muscle cells normally express nestin (3). Immortalization of primary CNS and non-CNS cells with the oncogene *SV40T* results in nestin expression only in cell lines derived from neuroectodermal cells (15). It thus appears that in neuroectodermally derived cells, nestin, in addition to the transient normal embryonic expression pattern, specifically reappears following transformation, an observation which supports the proposed relationship between gene expression patterns in CNS tumor cells and cells of the developing CNS.

Nestin and Diagnosis of CNS Tumors. There are several aspects to the use of nestin in CNS tumor diagnosis. First, the immunostaining is distinct and localized to the cytoplasm, which facilitates identification of positive cells. Second, nestin immunoreactivity in the nonneoplastic brain is very low, occurring only in endothelial cells. Third, the absence of nestin-positive tumor cells in the metastases and its frequent presence in anaplastic gliomas may assist in the differentiation of primary from metastatic tumors, particularly when small stereotactic biopsies are being used for diagnosis. The fact that a large proportion of highly malignant gliomas express nestin, as compared to the less malignant forms (*e.g.*, glioblastomas *versus* pilocytic astrocytomas), also suggests that nestin expression may relate to the degree of malignancy within the glioma group. High levels of nestin expression, in particular in glioblastomas, have also been reported by Tohyama *et al.* (14). Fourth, among the PNE tumors we observed nestin-expressing as well as nonexpressing tumors. Expression in PNE tumors has also been reported by Valtz *et al.* (16) and by Tohyama *et al.* (14). The different levels of nestin expression may be useful in the characterization of this tumor class, which is currently considered a heterogeneous and complex group (2, 27, 28). Currently, PNE tumors are primarily classified simply on the basis of their undifferentiated and variable morphology (29) (see Ref. 25 for an overview). GFAP, neurofilament, synaptophysin, and desmin immunoreactivity have been used to characterize PNE tumors and to indicate their differentiation potential (see Ref. 25). Considering that nestin expression during normal CNS development precedes GFAP and neurofilament expression (3, 8), it is possible, at least in a schematic model, that nestin expression in PNE tumors may be correlated with a more undifferentiated state, whereas coexpression of nestin and GFAP or neurofilament may define tumors in an intermediate differentiated state, while the expression of GFAP or neurofilament alone would define the most differentiated forms.

To improve the diagnosis of CNS tumors it is important to rely on objective criteria such as expression patterns of RNA or proteins from characterized genes. By improving our knowledge of such gene expression patterns we may be able to correlate this information with the biological aggressivness of the tumor or its sensitivity to various forms of therapy. With its predominantly early CNS-specific expression in normal tissue and expression particularly in malignant CNS tumors, nestin is one candidate for this type of analysis. The data show that CNS tumor cells can reexpress a gene normally active during CNS development. This supports the notion that the mechanisms controlling gene expression patterns are likely to be shared between developing and transformed cells.

Further investigations of larger numbers of tumors will reveal more precisely the role nestin will play in the clinical diagnosis of CNS tumors, but the results presented here demonstrate that nestin has a potential for joining the ranks of the other intermediate filaments, like neurofilament and GFAP, in providing useful information in tumor diagnosis.

ACKNOWLEDGMENTS

We wish to thank Dr. Magdalena Hartman for supplying sections of fixed, adult brain tissue; Drs. Ron McKay and Martha Marvin for the generous gift of anti-nestin antisera; Drs. Olle Johansson and Marita Hilleges for advice on immunofluorescence techniques, and Inga Maurin for preparing the tissue sections.

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Cancer Research **is sponsored by the American Association for Cancer Research, Inc. and receives major support from the American Cancer Society, Inc. Publication costs are also met by a grant from the Elsa U. Pardee Foundation.**

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